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STUDIES IN ABSORPTION.

B. H. Buxton and J. C. Torrey.

(*From the Laboratory of Experimental Pathology, Cornell University Medical College.*)

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I. Absorption of inert particles 5

II. Absorption of bacteria (typhoid) 18

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ABSORPTION FROM THE PERITONEAL CAVITY.*

PART I. — *Absorption of particles in suspension.*

B. H. BUXTON AND J. C. TORREY.

The present communication is the first of a series of papers, all of which deal with the subject of the absorption of substances in suspension from the peritoneal cavity. The ultimate object of the investigation concerns the method by which the organism disposes of bacteria injected into the peritoneum; and each of the papers is so intimately connected with the others that no one can be considered complete without the other four. The five papers may, therefore, be regarded as one complete study, and references in one paper may relate to topics discussed in later portions of the study.

The papers are:—

I. Absorption of inert particles.
II. Absorption of bacteria (typhoid).
III. The paths of absorption. Function of the diaphragm.
IV. The paths of absorption. Function of the omentum (inert particles).
V. The paths of absorption. Function of the omentum (typhoid bacilli).

I. ABSORPTION OF INERT PARTICLES.—In the present communication we shall confine ourselves to the question of absorption from the peritoneal cavity of guinea-pigs of insoluble particles, such as lampblack. The subject matter may be divided into two parts: *A.* Examination of the peritoneal fluid; *B.* Examination of the organs.

We may say briefly in advance:

1. Some of the particles remain in the peritoneal cavity and are there taken up first by the polynuclear leucocytes and later by the mononuclear macrophages.

2. Some of the particles leave the peritoneal cavity and

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* Received for publication April 5, 1906.

very rapidly reach the organs where they may be found in immense numbers.

A. The Peritoneal Fluid.—The French authors, with Metchnikoff at their head, tell us that if fluid is withdrawn from the peritoneal cavity of a normal animal—guinea-pigs are generally used—the fluid contains a considerable number both of lymphocytes and polynuclear leucocytes. Kanthack and Hardy further divide the former into the small lymphocytes with deeply-staining nuclei and scarcely any cytoplasm, and the larger mononuclears with vesicular, pale-staining nuclei and abundant non-granular homogeneous cytoplasm—their "hyaline cells." Kanthack and Hardy are also of the opinion that the polynuclear leucocytes of the fluid are all eosinophiles. They speak of the eosinophiles as cells of the body cavities as opposed to the ordinary polynuclears, which are distinctively cells of the blood under normal conditions.

Shortly—in a few minutes—after injection of particles in suspension into the peritoneal cavity (and the same applies to fluids of any kind—serum, bouillon, salt solution, etc.) the polynuclear leucocytes and hyaline cells almost entirely disappear from the fluid, although the number of small lymphocytes is hardly, if at all, affected, and the period of hypoleucocytosis or leucopenia sets in.

Metchnikoff ascribes the leucopenia to destruction of the cells and uses the term "phagolysis" to express it. Pierallini maintains that after injection of particles or fluids large numbers of the leucocytes are deposited on the omentum, many of them showing signs of degeneration. At the same time masses of fibrin are also found on the omentum; clear evidence that considerable numbers of leucocytes have been destroyed. Pierallini considers that there is certainly a partial destruction of leucocytes during the period of leucopenia, and most of the French authors take this view.

It may be mentioned here, in anticipation of what we shall have to describe more fully later on, that we also have frequently found deposits of fibrin on the surface of the omentum after intraperitoneal injections, but some recent discussions, reported in the "Comptes rendues, *Société de Biologie*, 1903," seem to throw some doubt on the accepted view that fibrin ferment is liberated on the destruction of leucocytes. Arthus, in particular, expressed himself as very strongly of the opinion that the fibrin ferment is an excretion of leucocytes excited to special activity, and is a product of the living cell only. Dastre, Stassano, and others agreed with him in the main. However this may be, it seems in view of these opinions hardly safe to assume that because there is deposition of fibrin on the omentum, therefore there has been phagolysis.

That there is phagolysis at all has been strenuously denied by some. Durham convinced himself that the leucopenia is due solely to clumping or balling of the leucocytes, in which condition they are swept by peristalsis towards the omentum and other peritoneal surfaces on which they are deposited. Consequently they are not brought up by the capillary pipette used for withdrawing the fluid from the peritoneal cavity, and from an examination of the fluid alone one would be led to suppose that the leucocytes had been destroyed. Lambotte and Stiennon maintain that leucocytes are by no means so fragile as is usually supposed, and refuse to believe in the doctrine of phagolysis.

The period of leucopenia lasts from one to three or four hours, varying with the reacting power of the animal and the nature of the substance injected, after which fresh polynuclear cells make their appearance in large numbers and actively take up the particles. The polynuclears now arriving are the ordinary polynuclears of the circulation with finely granular cytoplasm. Ehrlich's description of their granules as "amphophile" or "neutrophile" has been objected to by Kanthack and Hardy, who maintain that the granules are distinctly acidophile, staining readily with eosin, and Durham has proposed to call the cells "microxycytes," a term which seems to us legitimate, and which we shall make use of in these articles.

The microxycytes can be readily distinguished from the true eosinophile cells, in which the granules are much larger and stain still more strongly with eosin. Durham calls these "megoxycytes."

Our own experiments have been made principally on guinea-pigs with intraperitoneal injections of lampblack. The ordinary moist water color is emulsified in salt solution and the suspension when examined under a high power of the microscope is seen to consist of minute particles with some coarser lumps which can be filtered off. The filtrate is sterilized and used for injection.

We have endeavored always to make up the suspensions to as nearly as possible the same density and have used three cubic centimeters for each injection. From time to time drops of fluid were withdrawn from the peritoneal cavity by means of a capillary pipette and examined both in hanging drops and smears stained with eosinate of methylene blue.

The appearances after such injections have so often been described in detail that we need only say in a general way that we have always found the period of leucopenia well marked, but the question as to whether there is phagolysis or not will be reserved for discussion in a later section.

For about an hour or two there is little evidence of phagocytosis in the fluid, although the few polynuclears which can be found usually contain pigment granules; but in from two to six hours phagocytosis becomes very and increasingly marked, the newly arrived polynuclear leucocytes (microxycytes) taking up the particles very actively, whilst here and there a macrophage can be seen, though as yet not often in active commission (Plate I., 1).

In twenty-four hours the large mononuclear macrophages are present in great numbers, many of them having englobed one or more of the microxycytes with their contents (Plate I., 2).

It is not easy to determine from an examination of the fluid if the macrophages take up pigment directly or only englobe the phagocytes containing the particles. In many of the macrophages may be seen intact polynuclears, whose cytoplasm is filled with pigment granules, and from these on, all stages of degeneration appear, until the polynuclear cells are dissolved entirely, leaving only the pigment granules scattered about inside the macrophage (Plate I., 3).

However, in earlier stages, about four to six hours, while the macrophages are still few in number, and before they appear to have begun to ingest polynuclears, a certain proportion of them contain pigment granules, so it seems probable that they englobe particles directly as well as indirectly by means of the polynuclears. This observation is confirmed by what we have noticed on the surface of the omentum, as will be described later.

Phagocytosis of animal cells. — Metchnikoff has attempted to show that the chief function of the polynuclears or "microphages" is to take up bacteria and inert particles, whilst for animal cells they show negative chemiotaxis, remaining inactive; the macrophages doing all the work of phagocytosis when foreign animal cells or fragments of cells are introduced into the peritoneal cavity. In some experiments with chicken blood corpuscles we have not found this to be altogether the case. It is true the microxycytes do not actually englobe the chicken corpuscles, except in isolated instances, but this appears to be due simply to the mechanical difficulty of ingesting such large objects. The corpuscles appear to allow of very active positive chemiotaxis, for in one or two hours after injection, before the macrophages have begun to come in to any extent and while the microxycytes themselves are still scanty, each microxycyte that is seen appears to form a center around which a group of corpuscles is formed. Moreover, the cytoplasm of that part of any corpuscle actually in contact with a microxycyte often appears eroded, as if the polynuclear is partially digesting it. Again, the nuclei of chicken corpuscles may often be found in the microxycytes, so that if the corpuscles are reduced in size by loss of their cytoplasm they can be ingested. (Plate I., 4 shows a typical appearance one hour after injection.)

As soon as the macrophages appear they become active at once and the few which can be found between two and six hours after injection are already stuffed full of chicken corpuscles in various stages of degeneration. The size of the chicken red cells is no hindrance to the phagocytic action of the macrophages, which can distend themselves in a most extraordinary way and take up a dozen or so of corpuscles at a time, although when once taken up, the englobed corpuscles rapidly degenerate and shrink to a fraction of their former size (Plate I., 5). The macrophages certainly appear to attack the chicken corpuscles with much more energy than they attack bacteria or inert particles, and this is readily understood since their natural function is to englobe and carry off worn-out leucocytes or red cells and probably other cells of the organism to which they belong, so that they would be expected to have more affinity for animal cells of any kind than for particles of an altogether different nature. Still, although we can agree with Metchnikoff and other French authors that the macrophages play the chief part in disposing of animal cells, we cannot admit that the microxycytes are an unimportant factor in the methods adopted by the organism for disposing of extraneous particles of animal matter.

B. The Organs. — The second section of this article is concerned with an examination of the organs of the guinea-pigs which have received an injection of a suspension of lampblack particles into the peritoneal cavity according to the method indicated on page 8. The object has been to determine principally how quickly the particles reach the organs, in what amount, and for how long a period they may remain in the tissues.

After injection the animals were killed at various intervals. Some of the organs were then removed, sectioned by the celloidin method and stained lightly with hematoxylin and eosin. The sections were always cut to the same thickness (fifteen microns); and in their examination only those particles which could positively be identified as lampblack were counted. The enumeration, too, was confined to those particles which were manifestly in the interior of the section.

On this account the sections were made of sufficient thickness, so that extraneous particles of dirt lying on either surface might be excluded.

Early in the investigation it was decided to confine the observations to the liver and spleen. At first the lung and kidney were also included. An examination, however, of sections of the normal lung revealed a large number of cells in the alveoli, which contained particles that could not be distinguished from lampblack. These cells have been called by the Germans "staubzellen." In the latter part of this section their function is described. The kidney was finally excluded because it was found after many examinations that exceedingly few particles find their way to this organ. In sections of kidneys from guinea-pigs, killed twenty-four hours after the injection of lampblack, a few particles were occasionally found in the plexus of capillaries within the Malpighian bodies, but at no other place in the organ. These particles were evidently being carried along in the blood stream and were not deposited in the tissue, as is the case in the spleen and liver.

Muscatello, in a similar study, used a suspension of carmine particles in preference to lampblack, for the reason that he found black pigment particles normally present in the lung, lymph nodes, liver, spleen, and kidney. This objection to the use of lampblack certainly holds good for the lung, but an examination of sections of the other organs from normal guinea-pigs has convinced us that these pigment particles occur so infrequently that, for the purposes of our investigation, the possibility of their presence or absence may be disregarded.

In the following table a count is given of the number of aggregations of particles found in twenty fields of a Leitz microscope, using a number 7 objective, a number 3 ocular, and the tube drawn out to one hundred and sixty millimeters. As will be seen, the intervals of time at which the guinea-pigs were killed, after the injection of the lampblack suspension, range from five minutes to twenty-four days. It will also be observed that the first series of pigs was controlled by a second.

TABLE I.

Aggregations of lampblack particles in the liver and spleen.

Time.	Series.	Liver.	Spleen.	Remarks.
5 minutes.	1	0	2	Practically no particles have reached the organs.
15 minutes.	1	9	13	Particles free, scattered. In the liver free particles in the larger veins.
30 minutes.	1	4	0	Fewer particles than in 15 minutes. None to be found in the spleen.
	2	2	0	
1 hour.	1	0	0	A few scattered particles in the larger veins.
	2	0	0	
2 hours.	1	8	36	Granules for the most part free, a few inside leucocytes.
	2	30	21	
4 hours.	1	144	141	The great majority of the black granules inside leucocytes.
	2	154	101	
6 hours.	1	22	57	In both the liver and spleen there is marked drop in the number of particles from that of 4 hours. The aggregations of particles also appear smaller at this stage.
	2	43	45	
24 hours.	1	68	1,000	The spleen is crowded with large aggregations of black particles. A great many macrophages filled with granules in the pulp. The bile ducts of the liver contain a few particles.
	2	183	1,300	
48 hours.	1	66	205	A decrease in the number of particles, similar to that found in 6 hours.
	2	40	164	
3 days.	1	101	690	The black granules have increased in number, especially in the spleen.
4 days.	1	117	800	The conditions are very similar to those at the end of 24 hours. The spleen is again crowded. Some of the granules are free, but the great majority inside cells.
	2	260	1,200	
8 days.	1	50	215	Fewer particles in both the liver and spleen, otherwise about the same as 4 days.
	2	53	456	
16 days.	1	27	60	A further decrease in the number of particles.
24 days.	1	30	142	The spleen of the second series is crowded.
	2	126	2,000	

An examination of the table shows that until two hours after the injection of the lampblack suspension into the peritoneal cavity, the black particles are either entirely lacking in the organs or very few in number.

In the sections of the liver and spleen of the animal killed after five minutes there are practically no particles to be found in the tissues, nor in the veins and lymph spaces is there more than a suggestion of their approach. After fifteen minutes, however, there is no doubt of the presence of free aggregations of lampblack granules in the capillaries of the liver and spleen. In some of the medium-sized veins, too, a large number of free particles may be found. In no instance had they been ingested by leucocytes.

Turning now to the sections from pigs killed in thirty minutes, we find that no particles are to be found in the spleen and very few in the liver. In the larger blood vessels there are also fewer particles than in fifteen minutes. This is still more strikingly the case in one hour. It is, in fact, only after two hours that the lampblack particles can be found in any abundance in the liver and spleen. At this time the black granules are for the most part free, although here and there a leucocyte may be seen which contains a few. This observation agrees in the main with that of Muscatello. He injected a suspension of carmine particles into the peritoneal cavity of dogs and found that they appear in the liver and spleen after an interval of one and a half to two hours, partly free and partly within the leucocytes of the capillaries and venous sinuses. He was not able, however, to discover the presence of the carmine particles in these organs in twenty minutes to one hour after injection. In our sections of the fifteen-minute stage there is no doubt that the lampblack has reached the liver and spleen, but as yet in very small amount. The particles, too, are lying in a free state within the lumen of the capillaries and larger blood vessels.

As will be observed in the table, after four hours the number of the particles has increased considerably. They are apparently somewhat more numerous in the liver than in the spleen. Some of the granules are still free and scattered in the capillaries, but the great majority have been taken up by leucocytes. In some instances the particles seem to be also inside the liver cells, although it is difficult to be sure of this on account of the thickness of the sections.

As regards the sections from the animals killed after six hours we find a marked decrease in the number and size of the aggregations of particles in the liver. Since our conclusions are based on the results of an examination of only two guinea-pigs at each stage, much stress cannot be laid on this circumstance, for which there is no obvious explanation, but attention may well be called at this point to the remarkable uniformity of the increase and decrease of the number of granules at the various stages in both the series. This seems to be more than an accidental coincidence.

In twenty-four hours the number of granules has increased to some extent in the liver, but enormously in the spleen. This organ is crowded with large aggregations of black particles. The vast majority of these are in the pulp, contained within the macrophages, either in the lymph spaces, the capillaries or the veins. Here and there in the follicles, however, a few particles may be found, generally enclosed in cells. In the liver we find by far the greater number of particles inside wandering cells. Only a few are free, or inside the hepatic cells and the endothelial cells of the larger blood vessels. Some of the black granules, furthermore, may be seen either inside the lumen of the bile ducts or between the duct cells. This observation is of interest in the light of the suggestion of Siebel, that the bile ducts are a possible path of excretion of injected particles.

The most striking feature in the examination of the forty-eight-hour sections is the marked decrease of the number of black granules in both the liver and spleen. This is especially the case in the latter organ. The next stage of four days presents a condition which is practically identical with that at the end of twenty-four hours. The spleen is again crowded, and the aggregation of particles are large and free or contained within cells, probably macrophages for the most part.

From eight days to two months in the first series there is a decrease in the number of granules in the liver, and also to some extent in the spleen. In the second series the spleen was found to be crowded at the end of twenty-four days. This may be accounted for on the ground that this particular pig received a somewhat larger injection of lampblack suspension than the others. In all the later pigs the masses of granules are much larger than in the earlier ones, and the great majority are contained within large wandering cells, the macrophages, although here and there free granules may be found. It will also be observed that the sections of the spleen are far more crowded than those of the liver.

The decided and uniform decrease in the number of the black granules at the end of six hours and again in forty-eight hours is noteworthy. There has apparently been an excretion of the foreign particles at these periods, but by what pathway this has occurred we have not attempted to determine. It should also be borne in mind, however, that particles are continually being transported from the peritoneal cavity to the organs, and the amount of this influx may vary with the different periods.

The question of the fate of particles injected into animals has been the subject of many investigations. As long ago as 1867 Hoffmann and von Recklinghausen injected carmine and vermilion into the circulation. They found that these particles are soon deposited in the various organs, such as the spleen, lymp nodes, and the marrow, thus quickly disappearing from the blood and lymph. Instead of disappearing rapidly from the organs, as is the case with bacteria, these particles remained in the interstitial tissue of the glands for weeks, and were not found to be eliminated by the urine or bile. Similar observations have been made by Ponfick.

Siebel confirmed these observations and discussed the manner by which the particles might ultimately be disposed of, concluding that the principal channels of excretion are the lung, liver, and tonsils, and possibly the follicular structures of the small intestines. In the lung, the particles are taken up by the leucocytes and these in turn wander into the alveoli, from whence they are thrown off by the sputum. A similar process is carried out under normal circumstances by the "staubzellen" of the Germans. These are wandering cells in the alveoli, which, after picking up particles of dust deposited in the lungs, are carried out of the body in the sputum. In the liver some of the particles, according to Siebel, are carried away by leucocytes and leave the body by way of the tonsils and possibly also through other surface follicular structures, such as the lymphoid patches of the small intestine. On the other hand, other leucocytes containing particles wander out from the capillaries into the surrounding tissue and become connective tissue cells. Thus the particles may remain in the organism for life. Finally, he observed that some particles did not appear to be taken up by the leucocytes at all, but gradually made their way into the bile ducts and were excreted through the gall bladder.

In the experiments which have been mentioned above, the foreign matter was introduced into the circulation. Other investigators have studied the fate of particles injected into the peritoneal cavity. Such were the experiments of Muscatello which are also very similar to our own. As has been stated, he injected a suspension of carmine particles into the peritoneal cavity of dogs and found that the granules quickly absorbed and in one and a half to two hours appeared in the liver and spleen either free or within leucocytes in the blood vessels. As has already been indicated, this is quite in accordance with our own observations. After six hours, according to Muscatello, many particles have appeared in the liver and also in the venous spaces of the spleen. In smaller amount the granules are also found in the lung, the pancreas, and the testicle. Maffucci, after the same period of time, has found the particles only in the liver and the spleen. He concludes that the position of the granules was the same in the liver, whether the injection had been made into the peritoneum or the jugular vein. When the particles are introduced into the circulation they reach the organs with great rapidity. Werigo, after injecting carmine into the ear vein of rabbits found the particles in the liver and spleen in two to four minutes. The end result, however, is the same whether the injection be made intraperitoneally or intravenously. The difference is merely one of time.

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ABSORPTION FROM THE PERITONEAL CAVITY.*

PART II.—*Absorption of typhoid bacilli.*

B. H. BUXTON.

It was shown in the first of this series of papers that particles of lampblack injected into the peritoneal cavity of guinea-pigs soon find their way to the organs, more particularly the liver and spleen, where they may occur in large quantities. This line of investigation has been followed up with typhoid bacilli, with results analogous to those obtained with lampblack. The bacilli very rapidly reach the organs, where they appear to be deposited in such a way that they are not washed out with the blood on bleeding the animal to death.

Similar experiments have often been carried out after intravenous inoculations, but records of what occurs after injection into the peritoneal cavity are scanty, and there has apparently been no attempt at systematic investigation.

The most important studies which have been made on the fate of bacteria after injection into the circulation are probably those of Werigo and Bail.

Werigo[1] injected anthrax bacilli into the jugular vein of rabbits and killed the animals at varying intervals of time after inoculation. By sectioning the organs and counting the bacilli in the sections, he was able to demonstrate that the bacilli were very quickly deposited in the liver, spleen, and lungs. Even in a few minutes after injection the bacilli had largely disappeared from the circulation, being held in the capillaries of the various organs, where they are already contained in the phagocytes. Other investigators have confirmed his observations, but some maintain that during the early stages the bacilli lie free in the capillaries for the most part, being only taken up by the leucocytes later on.

More recently Bail[2] has shown that typhoid and cholera bacilli after intravenous injection into rabbits and guinea-pigs

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undergo a similar fate. In a few minutes they are deposited in the liver, spleen, and marrow, from which they cannot be washed away on bleeding the animal to death. The bacilli may still be found in the organs for several days after inoculation. Bail in some cases also injected into the pleural cavity and from this point the bacilli very quickly found their way into the organs. Bail's methods differed from those of Werigo in that he took portions of the organs and plated them out, counting the colonies on the plates after twenty-four hours' growth, at 37° C.

Since typhoid bacilli do not hold Gram's stain, it is not easy to demonstrate them in sections, so Bail's method of plating out portions of the organs was adopted for the experiments detailed here. An attempt was also made to determine the bactericidal action of the peritoneal fluid itself. For this the usual practice of withdrawing small quantities of fluid from time to time after injection, by means of a capillary pipette, and determining whether the bacilli are dead or not by staining reactions or plating out a few drops, seems open to some objections, and it was considered advisable to attempt the withdrawal of the fluid on a large scale by washing out the peritoneal cavity.

Normal rabbits were used for the experiments, the dose being invariably one-half of an agar culture. The strain of typhoid used was of very moderate virulence, the dose being about the largest which could be used with safety, the object naturally being to inject as many bacilli as possible, yet at the same time to keep below the minimum lethal dose. Briefly the method of procedure for each rabbit was as follows: — A twenty-hour agar culture was emulsified in ten cubic centimeters of salt solution, and one-half or five cubic centimeters injected into the peritoneal cavity. After a stated time — one, two, four or more hours — the rabbit was etherized and bled from the carotid, some of the blood being defibrinated, and some allowed to clot for serum. After bleeding, and just as the rabbit was at the point of death, four hundred cubic centimeters of salt solution were run into the peritoneal cavity and immediately withdrawn again. This was called "wash water" and was used for plating out. Immediately after washing out, the abdomen was opened and portions of various organs taken out and plated. Having thus given a general survey, it will be necessary to enter more into details in order to understand exactly how the experiments were carried out.

1. The washing out. — As soon as the bleeding is finished the rabbit is covered with a sterile cloth and tied tightly down to the operating table by two broad bands passed over the chest and flanks. Through a slit in the cloth a cut is made in the skin of the abdomen and the tissue cleared down to the muscle. A small slit is now made in the muscle and a glass trochar pushed through the slit into the cavity; the trochar being attached to a rubber hose connected with a flask on a shelf above, containing four hundred cubic centimeters of salt solution. The salt solution is siphoned into the cavity, an operation which only takes about a minute, the rubber hose detached from the trochar, and the operating table, specially designed for this purpose, turned over so that the rabbit is now abdomen downwards, suspended by the broad bands. Below is a flask into which the contents of the peritoneal cavity are run through the trochar. It takes three or four minutes to empty the cavity, and about three hundred and fifty cubic centimeters can be recovered out of the four hundred run in. The wash water is at once put on ice to prevent any increase of the bacilli and returned to later on.

2. The organs. — The operating table is now turned back so that the animal lies in its original position. The abdomen is opened, and if there is any urine in the bladder its surface is seared and one cubic centimeter pipetted out. The organs, or suitable pieces of them, are then taken out, but since they have been bathed in a fluid which may contain a large number of bacilli it is necessary to sterilize the surface. For this purpose, the pieces as they are taken out are plunged for a moment into boiling water and at once put into a cold chamber. Repeated tests have shown that

this is sufficient completely to sterilize the surface, while leaving the bacilli inside the organ unhurt. Of the organs, the spleen, one kidney, a piece of the liver, and a piece of the lung are taken. The bile also is collected by means of a capillary pipette after searing the surface of the gall bladder. About one-quarter to one-half of a cubic centimeter is all that can be obtained as a rule. Finally one of the thigh bones is cut off and put on ice.

3. Preparation of material for plating.—The spleen is taken as a unit, and pieces of the other organs as nearly as possible of the same size. Each piece is dipped once more into boiling water, placed on a square of fine wire gauze (mesh sixty to the inch), and, after cutting up with scissors into small particles, is rubbed with a pestle through the wire gauze into a beaker below, fifteen cubic centimeters of salt solution being gradually poured over the surface (Bail's method). Since the whole operation can be conducted very rapidly the risk of contamination is slight, and has been found by experience to be a negligible quantity. By this method nearly all the organ cells can be washed through, leaving only small particles of connective tissue on the surface of the gauze. The lung, however, can only be partially rubbed through, so that pieces of this about three times the size of the spleen are taken. The content of each beaker is at once emptied into a sterile test-tube and put on ice until all the pulps are ready for plating. The marrow of the thigh bone is scraped out and treated in the same way.

4. Plating out the organs.—As soon as the rubbing down of the organs is completed, a sufficient number of agar tubes, each containing eight cubic centimeters, are melted and cooled to 50° C., in a water bath. By this time the larger particles in the organ pulps have subsided in the test-tubes, and from each tube two cubic centimeters of the supernatant fluid is pipetted out and distributed into two agar tubes, one cubic centimeter in each, for plating. The colonies on each plate, therefore, represent the number of bacilli in one-fifteenth of the spleen, or portion of the other organs. The defibrinated blood, serum, and urine, one cubic centimeter of

each, and the bile are also plated out and the plates all incubated at 37° C., the colonies being always counted after twenty hours.

5. Plating out the wash water. — The flask containing the wash water is well shaken up and one cubic centimeter of the fluid pipetted into nine cubic centimeters of salt solution — dilution of one to ten. Of the dilution of one to ten, one cubic centimeter is pipetted into nine cubic centimeters salt solution — dilution of one to a hundred — and so on up to dilution of one to one million. From each of these dilutions one cubic centimeter is plated out in eight cubic centimeters of agar, and, at the dilutions which are expected to yield the best results for counting, two plates are often made. After twenty hours' incubation the colonies are counted, and an estimation is made of the number of bacilli remaining alive in the peritoneal cavity. As an illustration, we may suppose that at dilution:

1 to 10	colonies	about 1,000
1 to 100	"	121 and 93
1 to 1,000	"	8 and 11
1 to 10,000	"	2 and 0
1 to 100,000	"	0

We may say roughly that we have ten thousand bacilli per cubic centimeter of the wash water, and since four hundred cubic centimeters of salt solution was run in, the total number of bacilli left alive in the cavity may be taken as of the order of 400×10,000=4,000,000. To estimate the number of bacilli which are injected, the remaining five cubic centimeters of the original emulsion — five cubic centimeters have been used for injection — are run into four hundred cubic centimeters of salt solution, from which dilutions are made and the number of bacilli estimated as described for the wash water.

It may be mentioned that agar tubes of the same size, eleven-sixteenths inch in diameter, were always used for the culture to be injected and a very large number of controls invariably gave nearly the same results, namely, four thousand millions per half culture. It was not necessary, therefore, to repeat the control experiments in every instance. The number of bacilli injected can always be reckoned as of the order of four thousand millions.

Experimental. — In a series of previous experiments[3] it was found that normal rabbit serum " in vitro " would invariably destroy about the same number — one million per cubic centimeter — of typhoid bacilli, and in view of this regularity it was anticipated that results obtained " in vivo " might show a somewhat similar constancy to those " in vitro." It was anticipated, therefore, that by sacrificing two or three animals at each interval of time chosen some general law might be laid down as to the number of bacilli left alive in the peritoneal cavity, and the numbers occurring in the organs at stated periods after inoculation. It very quickly became apparent, however, that no regularity of the nature of that observed " in vitro " was to be expected, so it was decided to sacrifice six animals at each interval of time and then work up the results into tables to see if any general conclusions could be drawn.

To avoid any necessity for referring back to what has already been said, it may be repeated that rabbits only were used, the inoculations being made intraperitoneally, and the dose one-half of an agar culture of typhoid bacilli of very moderate virulence. The number of bacilli inoculated was of the order of four thousand millions.

As a rule, the rabbits suffered little from the inoculations, although there was frequently a drop in temperature with some dyspnea and somnolence between two and four or five hours after injection, after which there was generally complete recovery with a slight rise of temperature over the normal.

Some rabbits, however, died at intervals of from two to twenty-four hours after inoculation. With these there was a decided drop in temperature, which remained low until death ensued; in other words, these rabbits showed no power of reaction. Animals of varying size were used from two and a half to four and a half pounds, but the majority were three to four pounds in weight. The actual size of the rabbits seems to have little influence on the results. Sometimes a large rabbit would die, and sometimes a small one, although, on the whole, the smaller ones are slightly more susceptible than the larger animals. The tables give in each case the weight of the rabbit; and from a study of them it will become obvious that the irregularities in the results are not due to irregularity in the size of the animals used. The results depend upon the reacting power of the individual rabbits, and this varies so much that it is impossible to say that there is any minimum lethal dose of living typhoid bacilli. Bail came to the same conclusion in the course of his experiments with cholera and typhoid bacilli.

The intervals of time at which the rabbits were killed after inoculation were at first taken at two, four, and six hours, and the accompanying tables show the results of this series of experiments.

Under the heading "wash" of column two in each table is given an estimation of the total number of living bacilli which could be washed out of the peritoneal cavity, and are, therefore, either free or contained within cells free in the fluid. This, however, by no means represents the total number which are actually alive in the cavity. There is usually an extensive deposit of fibrin on the surface of the omentum, and in this fibrin there may be entangled enormous masses of bacilli which show no signs of degeneration on staining, and are obviously alive. There is always, however, a relation between the number of living bacilli fixed on the omentum and those found free in the fluid. It is a remarkable fact, as will be brought out later on, that a microscopical examination of the omentum enables one to judge roughly of the number of bacilli which are likely to be found in the fluid; and on the other hand, according to the number found alive in the fluid one can say with a fair degree of accuracy what will be the microscopical appearances on the omentum. The results under the heading of "wash," therefore, are fairly comparable one with another. If there are few in the "wash" there will be few, and if there are many in the "wash" there will be many fixed in the omentum. In other words, the condition of the omentum does not influence the relative amounts under "wash" in the different experiments.

The figures under the organ headings give, in round numbers, the colonies on a single plate. In almost every instance two plates were made from each organ and the average taken. The numbers in the two plates vary so little that the second was practically superfluous.

From the figures we can make a rough estimate of the total number alive in each organ. One cubic centimeter of the fifteen cubic centimeters of pulp was plated out, and since the liver is about one hundred, and one kidney about ten times the size of the spleen, the figures can be multiplied by fifteen for the spleen, one hundred and fifty for one kidney, and fifteen hundred for the liver, to arrive at an idea of the order of the total numbers alive in each of these organs.

TABLE I.

Two hours.

	Weight. *lbs.*	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	1 cc. Blood.	About ½ cc. Bile.	Temp.
1.	4	350,000,000	1,050	525	20	25	20	12	0	Constant.
2.	3	8,000,000	1,500	200	1,500	500	20	200	10?	Drop 1½.
3.	3½	800,000	100	15	3	60	0	3	0	
4.	4	550,000	100	50	10	20	2	0	0	Drop 1½.
5.	4½	40,000	25	15	0	10	1	0	0	Drop 5.
6.	4½	0	0	0	0	0	0	0	0	Constant.

TABLE II.

Four hours.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	1 cc. Blood.	About ½ cc. Bile.	Temp.
1.	3¾	85,000,000	1,000	150	200	250	....	11	0	Constant.
2.	3⅛	70,000,000	20,000	2,000	125	2,500	25	125	1,200?	Drop 3.
3.	4¼	60,000,000	100	10	5	15	0	0	0	Drop 2.
4.	4½	12,000,000	5,000	3,000	2,500	800	....	3	2,500?	
5.	3	3,000,000	150	25	50	125	2	25	....	Rise 1.
6.	3¼	4,000	0	0	0	0	0	0	0	Constant.

TABLE III.

Six hours.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.	Bile.	Temp.
1.	3¾	80,000,000	15,000	1,000	700	700	10	200	65	Drop 3.
2.	4	65,000,000	25,000	200	500	250	....	1	0	
3.	4½	65,000,000	1,000	0?	....	100	0			
4.	3¾	10,000,000	6,000	4,000	450	200	5	60	1?	Drop 2.
5.	3½	450,000	75	450	35	150	0	0	0	Drop 2 and rise 2.
6.	3½	0	0	0	0	0	0	0	0	Constant.

A glance at the first three tables shows at once the striking irregularity which has already been commented upon, and it is obviously impossible to say that, at a given period of time after inoculation, a normal rabbit will have disposed of so many bacilli, and that there will be about so many left alive. It is evident, however, that the bacilli which are left alive have not by any means been confined to the peritoneal cavity. There are in most cases large numbers in the various organs; most as a rule in the liver, and after this, in the spleen and marrow. The blood contains comparatively very few.

Another point to be noted is that the numbers of bacilli still alive in the peritoneal cavity, on the one hand, and in the organs, on the other, bear some relation to each other, for if there are many alive in the cavity, there are also many in the organs, though there are two or three exceptions to this rule.

The power of destroying the bacilli varies very much with the different animals. This is most marked in two hours, the numbers still alive in the peritoneal cavity varying from three hundred and fifty millions to zero. Rabbits (Table I., 6, and Table III., 6) appear to have destroyed everything.

We have noted that there are always more in the liver than in the spleen. Bail observed this also after intravenous and intrapleural inoculation, and remarks that "The number of colonies in the plates makes it seem probable that the first organ to be invaded is the liver."

TABLE IV.

Sixteen hours.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.	Bile.	Temp.
1.	4	8,000,000	600	1,200	250	300	4	0	0	Drop 3.
2.	4½	1,000,000	25	30	0	0	....	0	110	
3.	4	350,000	150	1,500	40	300	5	0	60	Constant.
4.	4½	60,000	15	100	12	00?	0	2?	0	Rise 1½.
5.	3½	....	3	25	2	5	0	0	0	Rise 1½.
6.	3½	6,000	0	0	0	0	0	0	0	Constant.

TABLE V.

Twenty-four hours.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.	Bile.	Temp.
1.	3	2,000,000	15	15	25	15	1	20	0	Rise 3.
2.	4½	1,000,000	12	800	120	120	0	5	1	Drop 3½.
3.	4	400,000	3	150	10	15	1	6	....	Drop 3.
4.	3½	200,000	4,000	400	....	50	0	1	0	Rise 4.
5.	4	60,000	0	0	6	0	0	1?	10?	Drop 3.
6.	4	10,000	0	30	8	00?	0	0	0	Constant.

TABLE VI.

Forty-eight hours.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.	Bile.	Temp.
1.	3¾	1,000,000	3	200	?	30	0	0	....	Drop 1, sick.
2.	3¾	350,000	1	650	10	5	3	1	0	Rise 2.
3.	3½	300,000	2	10	30	1	2	0	0	Rise 1.
4.	4	25,000	0	20	1	0	0	0	0	Drop 1½ & rise 3.
5.	3½	8,000	0	0	0	0	0	0	0	Rise 2.
6.	3¼	3,000	0	0	0	0	0	0	....	Rise 3½ & N.

Tables IV., V., and VI. show the conditions in sixteen, twenty-four, and forty-eight hours after inoculation. The irregularities here are not quite so pronounced as in the first three tables, although sufficiently so to prevent any general conclusions being drawn as to the number of bacilli which one might expect to find alive at each interval of time. The main feature is that the relative position of the liver and spleen have changed. With one or two exceptions the spleen contains relatively more than the liver. This is somewhat analogous to the results obtained with lampblack (Part I.), so does not necessarily indicate that the liver possesses a greater bactericidal power than the spleen. There is probably a gradual shifting of the bacilli toward the spleen, and it is quite possible that the chief part of the destruction during the later periods actually occurs in the spleen, although the numbers of bacilli found alive there may be relatively greater than in the other organs.

These tables also show that the numbers of bacilli left alive in the peritoneal cavity and in the organs have decreased considerably over the earlier periods of time. How long the bacilli persist is a question which has not yet been followed up. Two days is the limit thus far. It is understood that all the animals referred to in the tables are alive at the time of each experiment.

Having shown, then, that so early as two hours after inoculation there may be a large number of bacilli already present in the various organs, it was natural to reduce the time in order to determine how quickly the organs might become invaded.

Tables VII. and VIII. show the condition of rabbits sacrificed in one hour and half an hour after inoculation. It is found that the organs may already be swarming with bacilli; the numbers being even in excess of those found in two hours, and the irregularities more pronounced.

TABLE VII.

One hour.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.	Bile.
1...	2¾	450,000,000	2,500	425	30	500	10	1,000	0
2...	2½	6,000,000	2,000	500	150	900	2	200	
3...	?	800,000	500	?	40	500	....	3	0
4...	3¾	200,000	3,000	1,500	150	250	5	5	
5...	3¼	12,000	0	0	0	0	0	0	0
6...	2¾	4,000	225	20	0	70	....	0	

TABLE VIII.

Half hour.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.	Bile.
1...	2¾	500,000,000	5,000	3,500	700	1,500	200	5,000	
2...	3¼	160,000,000	12,000	8,000	150	500	130	150	0
3...	2½	150,000,000	25,000	15,000	5,000	4,000	2,000	25,000	
4...	2¼	6,000,000	70	15	130	10	0	5	
5...	2¼	2,500,000	7,000	4,000	40	500	10	20	45?
6...	2½	40,000	5	2	2	1	0	2	0

Finally the whole operation was conducted as quickly as possible. Immediately after inoculation the rabbit was etherized, and bled from the carotid, the time between the inoculation and the stoppage of the circulation not exceeding ten

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minutes.. Table IX. gives the results with seven rabbits tested in this way.

TABLE IX.

At once.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Blood.
1	2¾	2,500,000,000	25,000	3,500	8,000	2,000	3,000	25,000
2	3½	2,500,000,000	25,000	2,000	3,000	8,000	1,200	15,000
3	2½	1,500,000,000	7,000	3,000	2,000	1,000	100	10,000
4	2½	1,200,000,000	15,000	1,000	3,000	400	300	10,000
5	4	350,000	0	0	0	0	0	0
6	2¾	300,000	0	0	2	0	0	50
7	3	1,000	0	0	0	0	0	0

The findings appear somewhat astonishing. In four cases the animals are literally swarming the bacilli, whereas in three cases almost all of the bacilli have already disappeared, none having apparently found their way into the organs. It may be mentioned here that in these three cases the disappearance of the bacilli is real and not simply apparent on account of errors in the manipulation. The results on the plates are confirmed by microscopical examination of the omentum. Everything in the three cases points to a sudden, one might say, explosive destruction of the bacilli, a destruction which must almost certainly be due to the bactericidal action of the body fluids, and not to the comparatively slow process of phagocytosis. Here we have something akin to the rapid destruction of typhoid bacilli by normal serum "in vitro," whereas in the first four instances there is apparently little or no immediate destruction by the body fluids. It seems probable that in the last three cases the whole bacteriolytic force of the fluid has been expended and that from now on there would most likely have been an increase in the number of bacilli left alive followed by a period of slower destruction in which probably phagocytosis would play the leading part. Rabbits 5 and 6 in the Tables I., II., III., are probably cases analogous to those in Table IX., 5, 6, 7, under discussion; only in two instances, Table I., 6, and Table III., 6, does there appear to have been complete destruction of the bacilli. In the other cases there are more in the wash and in the organs than "at once."

Not less remarkable than the explosive destruction in these three cases is the rapidity with which the organs of the other four rabbits have been invaded by the bacilli. Twenty-five thousand colonies on the liver plate indicates that there must be something like thirty to forty millions in the entire liver, and the numbers in the other organs are also astonishingly great. At this stage we find enormous quantities also in the blood, and a comparison of the various tables makes it appear as if the bacilli quickly enter the circulation and are deposited from the blood in the various organs, for shortly afterwards in one, two, or more hours, the bacilli are comparatively few in the blood, but are still found in large numbers in the organs. But even in the organs there is a rapid decrease, there being far fewer in from one to six hours than immediately after inoculation. A reference may be made here to Bail's work. He conducted a number of experiments "in vitro" with serum plus organ cells, and observed that in the presence of such cells — of liver, spleen, etc. — the serum to a great extent was deprived of its bacteriolytic action. These observations of his were perfectly correct. I have made numerous tests on this point, following Bail's methods, with results entirely in accord with his, but it would be superfluous to give the experiments in detail. Hoke[4] and others have also made such experiments and have attributed the loss in bacteriolytic power of the serum to absorption of complement by the organ cells.

Now, Bail, finding that, after intravenous or intrapleural injections, bacilli were deposited in the organs and remained alive there for a considerable time, concluded that in such situations the conditions were analogous to those "in vitro," and that bacilli, once they had reached the organs, found themselves in an environment of blood plasma plus organ cells, so were to a great extent protected from the bactericidal action of the blood.

It seems to me, however, that this conclusion is erroneous, for, as we have seen by the foregoing tables, the bacilli rapidly decrease in the organs at first. After the primary invasion of the organs there is evidently a rapid initial destruction of bacilli, followed by a later and slower process, but no evidence that the bacilli are better protected in the liver and spleen than in the peritoneal cavity itself. It is probable that in the organs, as well as in the peritoneal cavity, there is usually a rapid extracellular bacteriolysis at first, and that after this has expended its force, the phagocytes continue the process, but more slowly. This initial destruction may be of an explosive character (Table IX., 5, 6, 7) or somewhat slower, though still manifest within an hour after inoculation. There is apparently, however, a period from two to six hours during which the bacilli may show some increase in the organs. If we take from each table the three rabbits which show the largest number of bacilli in the organs, and are, therefore, fairly comparable, by averaging the totals we get the results given in Table X. The averages are only made out in round numbers.

TABLE X.

Averages at different periods of time. Three rabbits from each table (I–IX.).

	IX.	VIII.	VII.	I.	II.	III.	IV.	V.	VI.
	At once.	30 min.	1 hr.	2 hrs.	4 hrs.	6 hrs.	16 hrs.	24 hrs.	48 hrs.
Liver……	22,000	15,000	2,500	850	8,500	15,000	250	10	2
Spleen…..	4,000	9,000	500	250	1,700	1,800	900	450	300
Lung……	4,500	2,000	100	10	130	500	100	45	20
Marrow …	3,700	2,000	630	200	400	400	300	60	12
Kidney….	1,500	700	6	14	9	3	3	1	2
Blood……	17,000	10,000	400	70	40	20?	0	10	0

It appears from this table that during the first two hours there is a rapid decrease in the liver, followed by a considerable increase during the succeeding four hours. The period between six and sixteen hours has not been bridged, but probably in six hours the second period of high figures is at its maximum. The spleen shows very similar results, but it seems to take somewhat longer than in the liver for the bacilli to reach the initial maximum amount. The second period of decrease in the spleen is much slower than in the case of the liver, a point which has already been commented upon. The other organs will be dealt with later.

Since the number of bacilli in the organs varies so much from one animal to another, it cannot be maintained that these averages are very reliable, but it is hardly probable that the figures are purely accidental, and so far as it may be considered to have value, Table X. seems to support the argument that there is an initial rapid destruction of bacilli, which may be explosive or may be spread over an hour or so, the body fluids expending the whole of their bactericidal force upon it, after which there is nothing to hinder the remaining bacilli from multiplying for a time until the phagocytes have arrived in full force. We know that in the peritoneal cavity it takes five or six hours before the phagocytes have arrived in any large quantity, and no doubt the same holds good for the organs. The second period of decrease, however, may be due to the body fluids gradually recovering their bactericidal power.

It may, however, be contended that the initial decreases shown in Table X. are not real, but only apparent, since in the earliest stages there are enormous quantities of bacilli in the circulation. The blood remaining in the organs, therefore, might appreciably increase the numbers of bacilli found in the liver and spleen, under "at once," so that a considerable proportion of the colonies on the plates might represent bacilli which were in reality free in the circulation and not deposited in the organs. After thoroughly bleeding an animal, however, surprisingly little blood is left in the vessels or capillaries. The spleen pulps, indeed, are usually tinged with red, but the liver pulp scarcely shows a trace of it. Moreover, on centrifuging the pulps the red blood cells separate out into a distinct layer of their own, and it is then seen how very few there are, even in the spleen, as compared with the mass of organ cells. It seems unmistakably evident that the immense majority of the bacilli must be almost immediately deposited in the organs in such a way that they are not washed out in bleeding, and those which can be considered as free in the blood current represent an insignificant fraction of the total number on the liver and spleen plates, even in ten minutes after inoculation (Table IX.).

In the foregoing comments on the tables the liver, spleen, and blood have been dealt with, but it remains to consider the other organs and fluids.

Lungs. — In making up the averages for the lung, a very marked exception at two hours and another at four hours have been omitted. It is difficult to determine from Table X. if bacilli are deposited in the lung in the sense in which they appear to be deposited in the liver and in the spleen. In the earlier stages the numbers found in the lung are always considerably less than in the blood, and seem to rise and fall with the numbers found in the blood, although later than two hours there is an increase of bacilli in the lung, notwithstanding that by this time the blood hardly contains any bacilli at all. In all probability, therefore, there is a certain amount of true deposition in the lung, though to a less extent than in the liver or spleen. The lung pulps are usually tinged with hemoglobin to about the same extent as the spleen, and since the numbers of bacilli found "at once" in these two organs are fairly comparable, it may be that in both cases a large proportion are in reality free in the blood at this early stage. Tchistovich,[5] commenting upon the well-known fact that shortly after intravenous injection of streptococci (or other bacteria) into a rabbit there is marked hypoleucocytosis, maintains that this is because the polynuclear leucocytes englobe the cocci and become arrested in the capillaries of the lung. The lung is the only organ in which phagocytosis by the polynuclears is manifest. In the liver it is the cells of Kupffer which act as phagocytes, while in the spleen, marrow, and kidney there is practically no phagocytosis. If this observation is correct, and holds good for typhoid bacilli, it would account for the large numbers of bacilli found in the lung during the earliest stages.

Marrow. — One exception at four hours has been made in making up the average. That the marrow cells show marked changes in infection is well shown, but there is nothing in the tables to indicate that the marrow plays any specially important part in bacteriolysis. The question of the bacteriolytic action of marrow cells and extracts has been much disputed by various authors without any definite conclusions being reached, and it would be unprofitable to discuss the point here.

The kidney. — Bail, in his series of experiments already alluded to, never found many colonies on his kidney plates, and these observations are fully confirmed by our tables. The bacilli do not appear to be held firmly in the organ, and the comparatively large numbers found initially are probably due to the small quantities of blood in the pulps. Taking this for granted, it affords an indication of the numbers which should be deducted from the liver and spleen plates in order to determine what proportion of the colonies should be reckoned as free in the blood current. It is obvious from a study of the tables that the totals would only be affected to an insignificant extent, except "at once" in the case of the spleen, a further indication that the spleen is not invaded so quickly as the liver.

In connection with the kidney it may be mentioned that whenever the bladder contained urine, one cubic centimeter was plated out. The plates invariably remained sterile, except in one or two instances, where the urine appeared to have been previously infected. No typhoid colonies were ever found on the plates. Living typhoid bacilli, therefore, are not eliminated in the urine under the conditions of these experiments. This fact appears to lend some support to Metchnikoff's argument that bacteria are not eliminated by the kidneys unless there is some definite lesion. He discusses this question at length in "l'Immunité," but space forbids a recapitulation. Canon,[6] in a review of the literature on the subject, also comes to the conclusion that whenever the bacteria of invasion are found in the urine it always points to a lesion of the kidney.

The bile. — It cannot be said that the experiments afford any support to the contention that the bile is a regular channel for the excretion of bacteria. Only in two instances (Table II., four hours) were any large numbers of colonies found on the plates, and these are so exceptional that some error of manipulation must be suspected. Apart from these and three or four other doubtful cases, marked "?" in the tables, there are only three instances, one at six and two at sixteen hours, where the bacilli seem to occur in the bile.

Doerr[7] inoculated rabbits intravenously with typhoid bacilli, and always found bacilli present in the gall bladder after twenty-four and forty-eight hours. After intraperitoneal injections the bile was always sterile. But our own experiments show that, except in some instances where the bacilli are destroyed explosively intraperitoneally, injection into the peritoneal cavity is practically the same as intravenous injection in its immediate effects. The liver is very rapidly invaded in either case, so that the results obtained by Doerr are probably not reliable.

Serum. — In many instances one cubic centimeter of the serum was collected and plated out between one and two hours after bleeding. The plates were generally sterile, only comparatively few colonies appearing even when the defibrinated blood showed immense numbers.

In all probability the great mass of the bacilli are held in the clot and do not work out with the serum. Moreover, in one or two hours at room temperature the strongly bactericidal action of the drawn blood may have some influence. With the defibrinated blood this factor could hardly have produced any appreciable effect, since the blood was already on ice within five or six minutes after being drawn.

Temperature. — Up to one hour after injection there is practically never any change of temperature, although, as already remarked, there is usually a drop in two or three hours, which is followed later by a rise. But the temperature of normal rabbits varies so much that a drop or rise of one or two degrees is of little significance. Table I., 5, however, shows a drop of five degrees in two hours, and this rabbit would almost certainly have died. It has killed almost all the bacilli, and one might infer from this that if the bacilli are killed explosively by the rabbit, the rabbit is likely to be killed explosively by the toxins liberated. This may have been so in this instance, but the very next rabbit, Table I., 6, has killed all the bacilli in two hours, yet the temperature remains constant. Similar contradictory results are to be observed in the other tables, and one can only say that the temperature appears to give no clue as to what the result of the experiment will be. A glance forward, however, to Table XI. shows that there is always a marked drop in temperature when the animal succumbs.

Before concluding the article a table may be given showing the results obtained with six rabbits which died inopportunely. Only those are included the moment of whose death was observed. If for various reasons the manipulation could not be carried out at once, the rabbit was immediately packed in ice, so that there could not have been any material increase in the number of bacilli from the moment of death until the organs became thoroughly cooled down.

TABLE XI.

After death.

	Weight.	Wash.	Liver.	Spleen.	Lung.	Marrow.	Kidney.	Died in	Examined after death in	Temp.
1.	4½	4,800,000,000	125	0	300	00	350	17 hrs.	At once	
2.	4½	480,000,000	20,000	150	600	450	150	3 "	At once	Drop 5.
3.	3½	35,000,000	5,000	1,500	750	2,500	25	2½ "	1 hour	Drop 3½.
4.	3	6,000,000	1,500	1,200	40	700	2	2 "	3 hours	
5.	4½	6,000,000	1,500	250	60	200	3	2½ "	2 "	Drop 3½.
6.	3	2,500,000	100	300	50	3,500	0	23 "	At once	Drop 5.

If we consider the four animals which died very shortly after inoculation, it appears that with the exception of Rabbit

2 the number of bacilli left alive in the peritoneal cavity and in the organs are of much the same order as in the case of those killed in two and four hours, many of which appeared to be none the worse for the inoculation. There is no obvious reason why some should have died and others lived. Of the two rabbits which survived until the day after the inoculation, the most striking point of similarity is the very large number of bacilli in the marrow as compared with the other organs, but it is impossible with only two experiences to say if this has any significance or not. This similarity, too, is offset by the great differences in the number of bacilli found alive in the peritoneal cavity, a proportion of five thousand to six.

Nor does the table appear to afford much insight into the action of the endotoxins. It is generally accepted that if an animal dies within a very short time after inoculation of bacteria death is due to liberation of endotoxins, on account of the organism having destroyed immense numbers of bacteria. We might suppose this to be the case with XI., 4 and 5, but XI., 2, is swarming with bacilli, and one could hardly imagine that in this instance sufficient endotoxins have been liberated to destroy the animal, unless it is assumed that, the whole organism being quickly overrun with bacilli, some may have been killed off at a specially susceptible focus, such as a nerve center, where a comparatively little toxin being absorbed directly might be sufficient to destroy life. In all likelihood, however, it will remain a puzzle for some time to come why some rabbits can kill millions of bacilli and appear none the worse, while others die in consequence of having killed millions, and again why some become flooded with bacilli and live, while others die in consequence of being so flooded.

To sum up, it may be remarked that we have here a number of observations which in many respects appear contradictory and difficult to reconcile with the current theories of natural immunity. A discussion of their bearing upon these theories will have to be postponed until after a parallel series of experiments shall have been made with immunized animals. It may then be possible, with the help of observations on the paths of absorption from the peritoneal cavity and the part played by the omentum, to bring a certain degree of order out of the somewhat chaotic mass of facts obtained thus far.

CONCLUSIONS.

After intraperitoneal injection of typhoid bacilli into rabbits:

1. There may be an explosive destruction of the vast majority of the bacilli, few or none reaching the organs in such cases.
2. The destruction may take place more slowly, though still strikingly manifest within one hour after inoculation.
3. In the latter case large numbers of the bacilli find their way into the circulation almost immediately, being rapidly deposited from the blood in the various organs.
4. The organs vary greatly in their power of holding up the bacilli, the liver being invaded to the greatest extent.
5. The number of bacilli in the organs rapidly decreases again after the first few minutes up to about two hours after inoculation.
6. From two to six hours there is a considerable increase, probably due to the body fluids having expended all their energy, thus permitting multiplication of the bacilli.
7. From six hours up to forty-eight hours there is again a decrease, rapid in the liver, but more gradual in the spleen. The second period of decrease is probably due to the action of the phagocytes, although recovery of the bactericidal action of the body fluids may also be a factor.
8. Neither the urine nor the bile appear to be channels of excretion under the conditions of the experiments.
9. The variations of temperature between the time of inoculation and that of bleeding afford no indication of what the results of the experiments are likely to be.
10. Experiments with rabbit serum "in vitro" give no clue as to what will happen "in vivo."

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ABSORPTION FROM THE PERITONEAL CAVITY.

PART III. — *The function of the diaphragm.*

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In the preceding papers we have shown how inert particles, like lampblack, or typhoid bacilli, reach the internal organs with extraordinary rapidity after inoculation into the peritoneal cavity. The experiments on which these conclusions have been based were given in detail, but no attempt was made to explain by what paths the particles or bacteria reach the organs. Coincidentally with these experiments we have been endeavoring to determine this point also, and the present communication deals with our investigations along this line.

Since bacteria (typhoid bacilli) can often reach the organs and become deposited in enormous numbers in the liver, spleen, and marrow within ten to fifteen minutes after inoculation, and particles of lampblack with equal rapidity, although apparently not to such a great extent, it is necessary to suppose an initial rapid rush to the interior organs. It can hardly be supposed that the initial rush is altogether due to the action of phagocytes which have already picked up the particles or bacilli and are carrying them off. We have indeed shown in Part I. that particles of lampblack in the earliest stages lie free in the veins and capillaries of the liver, and it is not until after two to four hours that they are found, for the most part, in the phagocytes. We find, also, strong evidence that the particles on their way to the organs are carried in a free condition, for they can be found free in immense numbers in the lymph trunks and nodes of the anterior mediastinum, whence they can readily pass into the thoracic ducts and the blood current. The source of the anterior mediastinal lymphatic trunks can be traced to the lymphatics of the diaphragm, so it appears highly probable that the diaphragm plays an important part in absorption of particles from the peritoneal cavity. Since most, if not all, of the particles carried along the lymphatic trunks must pass through the lymph nodes connected with this system, it was thought that a study of the anterior mediastinal lymph nodes at various intervals after inoculation should prove of some interest.

The experiments have been conducted along two lines:

A. Examination of sections of the anterior mediastinal nodes.

B. Enumeration of the bacilli (typhoid) in the anterior mediastinal nodes by plating methods.

A. Sections of the anterior mediastinal lymph nodes.

If a guinea-pig is killed in five to fifteen minutes after injection of lampblack intraperitoneally, and the thorax opened, the anterior mediastinal nodes are found to be already quite black, and there is often a black line running along the anterior mediastinum on either side of the sternum in close proximity to the internal mammary vessels. The black line represents the anterior mediastinal lymphatic trunk. The most prominent and constant of these nodes are a pair, often three, in the first intercostal spaces. There are usually one or two other pairs lower down in the third or fourth intercostal spaces, but these are somewhat irregular both as to location and occurrence. These nodes will be designated as the "upper" and "lower" anterior mediastinals.

The sections on which this work is based were made by the paraffin method. They were cut for the most part six microns in thickness, and in many instances mounted serially. A variety of stains were used, such as hematoxylin and eosin, Gram's, and eosinate of methylene blue.

I. Sections of early stages.

a. Sections after injection of lampblack. — Although in fifteen minutes after injection of lampblack the nodes appear quite black, sections at this stage with a low magnification show that the particles lie in the peripheral afferent sinuses of the lymphatic channels, and the center of the node is only slightly pigmented (Plate II., 1). In the lower nodes all of the afferent channels are filled with lampblack. Under a high power of the microscope the great majority of these particles are seen to be free, only here and there do we find a macrophage containing black granules (Plate II., 3). In the inner and efferent sinuses a considerable amount of free pigment may be found, but a much larger proportion of the particles are contained within cells than in the afferent plexuses. The cells are for the most part macrophages. A few microxycytes may be seen, but they have taken as yet no part in the phagocytosis. The upper nodes are not, like the lower, pigmented throughout their periphery, but only in the region where the afferent ducts enter the gland. As with the lower nodes, however, the greater part of the lampblack in the efferent sinuses of the upper pair has been taken up by macrophages, whereas at the periphery most of the particles are free. Although the microxycyte reaction is beginning in the lower nodes, these cells have not as yet invaded the upper pair.

An examination of the afferent lymphatic ducts of the lower nodes shows that at this early stage all of the lampblack particles have come from the diaphragm in a free state and as such enter the nodes. In passing through the gland, however, some of the particles are seized upon by wandering cells and detained, whereas the major part passes on into the efferent ducts entirely as free particles. A photograph of one of these efferent ducts filled with free particles is shown in Plate II., 5. The same is true of the upper nodes. The particles enter and leave the nodes in a free state, but in the sinuses are partly ingested by cells.

b. Sections after injection of chicken red cells. — Chicken red cells injected into the peritoneal cavity of a guinea-pig also reach the nodes in fifteen minutes, but in much smaller quantity than lampblack. The red nucleated cells are almost entirely free and stain well. Here and there macrophages have begun the attack and various stages of ingestion may be found. An early stage of ingestion may be found in Plate VII., 1, on the left.

After an interval of one hour the afferent channels of the lower nodes are gorged with chicken cells. Most of these are free, some are clumped together in groups of two or three, whereas others are contained within macrophages, where they may be found in various stages of destruction (Plate VII., 1). As is the case with lampblack, sections of the upper nodes do not contain so many chicken cells as the lower. In the afferent sinuses many of these cells are being taken up by macrophages. In the inner sinuses there are only a few chicken cells and these are for the most part free. There are quite a number of microxycytes to be seen and some of these seem to be attacking the foreign cells in a manner which has been described as occurring in the peritoneal fluid, *i.e.*, the microxycyte forms a center of attraction around which are grouped a number of chicken cells. Although the afferent sinuses are crowded with chicken cells they are rarely met with at this stage in the efferent sinuses. Plate VII., 1, shows the afferent sinuses of a node one hour after injection. Most of the chicken red cells are free, but some are contained within macrophages.

c. Sections after injection of bacteria. — Turning now to sections of mediastinal nodes after injection of an emulsion of typhoid bacilli, we find at the end of an hour a somewhat different condition of affairs than that seen after the injection of inert particles or foreign cells. Instead of the greater number of bacilli being free in the afferent sinuses of the node, nearly all have been taken up by macrophages. A few bacilli have also been conveyed by these wandering cells into the inner lymph channels. Throughout the node a very small minority of typhoid bacilli are lying in a free condition. Microxycytes are present in large numbers, as was the case with chicken cells, but they seem to take very little part in the phagocytosis.

It is rather surprising to find that such large bacilli as anthrax can be absorbed through the diaphragm of a guinea-pig and reach the anterior mediastinal nodes in fifteen minutes or less. Even chains of four or five bacilli in a free condition have found their way into the lymphatics of the diaphragm. In the lower nodes these bacilli are present after fifteen minutes in much greater abundance than in the upper, but are confined in each case entirely to the afferent sinuses. Anthrax bacilli do not appear to be taken up by the phagocytes so readily as typhoid bacilli.

As would be expected staphylococci can be readily absorbed through the diaphragm. After one hour they are found in the afferent sinuses within the macrophages. A few cocci both free and in cells have also worked their way into the interior of the node. Microxycytes have been attracted in great numbers. In the afferent sinuses, where the cocci are present in abundance, all the available space is taken up by microxycytes. Although here and there a microxycyte may be found which has ingested several cocci, by far the greater number are clearly in the macrophages, possibly because these cells have been able to seize upon the cocci before the arrival of the microxycytes. It may be said, however, that the microxycytes which have wandered into the node show rather more marked phagocytic activities when the inoculation has been made with staphylococci than is the case with typhoid bacilli.

2. Sections of later stages.

a. Sections after injection of lampblack. — In Plate II., Fig. 2, is shown a photograph of a section of an upper mediastinal node under low magnification, twenty-four hours after injection of lampblack. It presents a very different appearance from that of the fifteen-minute stage. Much less pigment is found in the afferent sinuses, and what there is appears to be almost entirely contained within macrophages and microxycytes. The efferent sinuses on the other hand are now well filled with cells stuffed with lampblack.

As is shown by a high power of the microscope (Plate II., 4), the macrophages often contain so many particles that little or none of the protoplasm of the cell can be seen. The microxycytes have invaded the nodes in large numbers. Nearly every one has taken up a few particles, but none have gorged themselves after the manner of the macrophages. In addition to the particles, some of the macrophages have also ingested microxycytes. A few free particles may still be found, but most of the lampblack is leaving the node within wandering cells. Although, as will be shown, foreign cells and some forms of bacteria disappear from the nodes within a short time, lampblack remains there very much longer. Even at the end of two months both the upper and lower nodes appear quite as black as at any previous stage.

b. Sections after injection of chicken cells. Later stages.— As regards the later stages with chicken cells, we find that at the end of eight hours almost every cell has been taken up by the macrophages. The efferent sinuses contain far more than the afferent. In the latter the macrophages have almost entirely digested the chicken cells. In many there is little left except the nuclei. The macrophages in the inner sinuses have stuffed themselves to a remarkable degree. They frequently contain from eight to fifteen of these cells, which are in all stages of destruction (Plate VII., 2). This digestion has progressed so far at the end of forty-eight hours that only occasionally a chicken cell may be found. Microxycytes are quite abundant, especially near the periphery of the node, but they take comparatively little part in the destruction of the foreign cells.

c. Sections after injection of bacteria. Later stages.— In four hours most of the typhoid bacilli have left the afferent sinuses and are found in abundance in macrophages within the inner and efferent lymph channels. Many of these macrophages which contain bacilli are vacuolated and stain poorly; a form of degeneration which is especially noticeable among those at the periphery of the node. At this stage, that is to say, in four hours after the injection of the typhoid bacilli, microxycytes are much more in evidence than after injection of inert particles. A few of these cells are already contained within macrophages, but those which are still free and presumably in an active condition seem to take little part in the phagocytosis. It is only here and there that a microxycyte containing typhoid bacilli can be seen.

Anthrax and staphylococci were found to have almost entirely disappeared from the nodes in four or five hours. Although apparently differing from typhoid in this respect, sufficient experimentation has not been carried on with these bacteria to permit us to lay much emphasis on this observation.

Colloidal platinum. — A few experiments were made with colloidal platinum prepared by Bredig's method of striking an arc between two platinum electrodes under pure water. The particles are ultra-microscopical, but on addition of an electrolyte increase in size and gradually precipitate out. One hour after injection of the colloidal solution very few particles could be found in the nodes, but this may be due to the majority of them being still of ultra-microscopic size. In four hours they are plainly visible and have been entirely taken up by macrophages. The position of the particles in the cells may be observed to much better advantage when this agent is used instead of lampblack, since it is semi-transparent, and the cells do not become so packed. Colloidal platinum evidently has little power of attracting microxycytes, for few of these wandering cells are found in the node.

B. Enumeration of bacilli by plating methods.

In addition to the section work, some experiments were made by plating out the anterior mediastinal lymph nodes after injection into rabbits of typhoid bacilli. The rabbits were the same as those used for the experiments already detailed in Part II., but in the earlier experiments the idea of testing the lymph nodes had not occurred to us, so that only from three to five results of each period can be recorded.

The lymph nodes were treated precisely as has been described for the spleen and other organs. The upper pair were picked out and rubbed through a fine wire gauze with fifteen cubic centimeters of salt solution. Since it is not easy to pick out the lower pair of nodes, the tissue where they lie was cut out and treated in the same way. Of the fluid one cubic centimeter was plated out and also fractions of one cubic centimeter. From the colonies on the plates the total number of bacilli in the nodes could be roughly estimated. Since there is always a little uncertainty even with the upper pair, whether the nodes have been completely removed or not, the individual figures must not be taken too literally, but, regarded as a whole, there is no doubt that the lymph nodes may contain enormous quantites of bacilli, the upper pair always containing far more than the lower. It has been said that after injection of inert particles or bacteria into guinea-pigs sections appear to show that in the earliest stages the lower nodes are invaded to a greater extent than the upper. The accompanying tables under " at once " and " half hour " seem to contradict this statement, but it must be remembered that the upper nodes are always much larger than the lower; a disproportion, moreover, which is more marked in the rabbit than in the guinea-pig. It cannot be supposed that the small pieces of muscle and connective tissue attached to the nodes after cutting out can materially influence the results. Our sections show that lampblack, chicken cells, and bacteria are strictly confined to the lymph nodes and trunks, so that the possibility of large numbers of typhoid bacilli occurring in the tissues adjacent to the nodes may be excluded.

TABLE I.

Estimated number of bacilli occurring in the lymph nodes after intraperitoneal injection into a rabbit of half a culture of typhoid. Anterior mediastinal lymph nodes, upper pair.

Rabbit No.	IX.	VIII.	VII.	I.	II.	III.	IV.	V.	VI.
	At once.	30 min.	1 hour.	2 hours.	4 hours.	6 hours.	16 hours.	24 hours.	48 hours.
1...	25,000,000	6,000,000	....	....	....	....	1,500,000	....	5,000,000
2...	....	6,000,000	200,000	2,000,000	4,500,000	....	....	10,000,000	
3...	500,000	5,000,000	....	....	....	....	15,000,000		
4...	....	....	3,000,000	300,000	....	1,200,000	2,000,000	3,000,000	60,000
5...	....	1,500,000	60	....	300,000	50,000	75,000	....	350
6...	....	600	12	....	13	0	0	200,000	0
7...	120	....	....	6,000,000	25,000,000				

TABLE II.

Estimated number of bacilli occurring in the lymph nodes after intraperitoneal injection into a rabbit of half a culture of typhoid. Anterior mediastinal lymph nodes, lower pair.

Rabbit No.	IX.	VIII.	VII.	I.	II.	III.	IV.	V.	VI.
	At once.	30 min.	1 hour.	2 hours.	4 hours.	6 hours.	16 hours.	24 hours.	48 hours.
1...	400,000	100,000	....	....	....	....	20,000	....	10,000
2...	....	15,000	10	12,000	12,000	....	....	250,000	
3...	25,000	25,000	....	....	....	....	10,000		
4...	....	....	50,000	100	....	1,000	5,000	120,000	600
5...	....	2,000	0	....	150	3,500	100	....	0
6...	....	0	0	....	0	0	0	0	0
7..	0	....	....	250,000	600,000				

Table I. gives the estimated number of bacilli in the upper pair of nodes at various intervals of time, and Table II. the corresponding numbers in the lower pair.

The rabbit numbers correspond with those of the previous tables given in Part II. For instance, the upper nodes of Rabbit No. 1 "at once" contain twenty-five million bacilli, and a reference to Rabbit No. 1 of Table IX. in Part II. will show the results which were obtained with the organs of this rabbit. The only exceptions are numbers 7 in two hours and four hours, which were not brought into the previous tables. It will be remembered that the tables in Part II. were arranged in such a way that the topmost row began with the highest figures, the figures gradually decreasing to the lowest row. On the whole, the same decrease can be noticed in these two tables, so it is evident that there is some relation between the numbers of bacilli found in the organs and those found in the lymph nodes. If the organs contain many bacilli the lymph nodes also contain many, and if there are few in the one case then there are few also in the other. To this general rule we find only two exceptions, both of which are rather marked, at one hour (II.) and at sixteen hours (I.). In these cases we may suspect that the lymph nodes have not been entirely cut out. In Table

II. variations are more in evidence, but with the lower nodes the uncertainty as to whether or not they have been actually included in the material removed is much greater than with the upper pair.

In the previous experiments on the number of bacilli occurring in the organs it was noticed that after a marked decrease in two hours there appeared to be an increase of the bacilli in the four and six hour periods, followed again by a decrease in sixteen hours. There is no evidence of the secondary rise in Table I., the numbers in the nodes after four and six hours being on the whole fairly comparable with those at the same level during the earlier periods.

It is remarkable that the three later periods of sixteen, twenty-four, and forty-eight hours afford such high figures. These are the periods at which the spleen always, or very nearly always, shows a decided preponderance over the other organs in the numbers of bacilli it contains. It was observed in Part II. that this fact probably indicates a gradual shifting of the bacilli from the other organs toward the spleen, and this appears to hold good also for the mediastinal lymph nodes. But in this case the process is probably not so much a general shifting of the bacilli toward the lymph nodes as a holding up by the nodes of fresh supplies from the peritoneal cavity. After the initial rush in which the lymph nodes are overwhelmed, as it were, they probably settle down to their work, and later on more effectually prevent any further supply of bacilli from reaching the internal organs. In the lymph nodes, to judge of the appearance in sections, the macrophages take up and gradually digest the bacilli. This at any rate seems to be the probable explanation of the comparatively large numbers of bacilli occurring in the lymph nodes during the later stages, the macrophages probably remaining largely in the lymph nodes, where they digest the bacilli apparently more slowly than they digest chicken red cells, which in forty-eight hours have almost entirely disappeared.

The early infection of the anterior mediastinal nodes after intraperitoneal injection has not been recognized until lately.

Muscatello[1] in 1895 observed that in five to seven minutes after injection of carmine into the peritoneal cavity of dogs the mediastinal lymph nodes become colored. He expresses himself as surprised to find that this phenomenon has not been more frequently noticed, for he can find but one reference to it in the literature, that of Dunbar and Remy in 1882.

Durham[2] found that Indian ink reaches the anterior mediastinal nodes in about eight minutes after injection intraperitoneally in guinea-pigs, while cholera bacilli may be found in six minutes. The nodes are not connected with the omental lymphatics but with the diaphragmatic. Our experiments are quite in accord with the above and we may take it as fairly well established that there is a vigorous initial rush of particles or bacilli to the anterior mediastinal lymph trunks and nodes by way of the lymphatics of the diaphragm.

The next point for discussion is, "How do the particles pass from the peritoneal cavity into the lymphatics of the diaphragm?"

Von Reckinghausen in 1862 considered that he had demonstrated the presence of stomata in the diaphragm, and since then the existence of stomata in the diaphragm has been generally accepted and is taken for granted in the current text-books. According to this view it is easy to see how particles could pass through such openings into the lymphatics, since the peritoneum could be regarded simply as an enormous lymphatic channel in direct communication with the lymphatics of the diaphragm towards which peristalsis and the pumping action of respiration would direct the flow of lymph.

But recently the existence of the stomata has been strenuously denied. In the first place, Sabin[3] has very clearly demonstrated that the lymphatic channels and ducts form a system "sui generis," which has no connection ontogenetically with the connective tissue spaces or the large serous cavities. The lymphatics develop by a budding out from the endothelium of the subclavian veins and spread a network of ducts all over the body by progressive budding out of blind sacs. Sabin regards the lymphatics as modified veins. One would not expect, therefore, the lymphatics to be in direct open communication with the peritoneal cavity, and Muscatello, as a result of his investigations, concludes that there is always a membrane covered on either side with lining cells between the lymphatics of the diaphragm and the peritoneal cavity. Nevertheless, he considers that the diaphragm is the only point of absorption from the cavity, and, since in the earlier stages he finds many of the particles free in the anterior mediastinal nodes, suggests that leucocytes after ingesting particles force their way through the membrane and are immediately followed by a rush of free particles which continues until the space closes up again, just as Thoma found a rush of red cells might follow the passage of a leucocyte through a capillary wall. In this way the smaller particles pass through in a free condition, the larger ones being taken up by the phagocytes.

MacCallum[4] agrees with Muscatello and, as a result of very careful and minute observations on the diaphragm, concludes that its lymphatics are not in direct communication with the peritoneal cavity, but that in places there is nothing between the two except the endothelium of the lymphatics on the one side, and the lining of the peritoneum on the other, with an exceedingly fine basement membrane between the two layers of cells. At such points the lymphatic channels are enlarged, forming lacunæ, and the free particles (carmine or Indian ink) can be forced through between the lining cells from the peritoneal cavity to the lacunæ of the lymphatics by the pumping action of the diaphragm. However, MacCallum accords the chief rôle to the phagocytes which take up particles in the peritoneum and wander through to the lacunæ. His drawings show that he considers the polynuclears (microxycytes) to be the chief agents in phagocytosis.

As to the presence or absence of a direct connection between the peritoneal cavity and the lymphatics of the diaphragm, we have not been able to decide from a histological standpoint. From its physical aspect, however, it seems hardly possible that such relatively large objects as chicken cells or a chain of four or five anthrax bacilli could force their way through two layers of cells and a basement membrane and reach the anterior mediastinal nodes within fifteen minutes. There is no doubt that in this early stage, and in all probability in the later ones also, the chicken cells reach the lymphatic ducts in a free condition. It is not until they have reached the lymph nodes that their destruction by macrophages begins. The microxycytes are rarely able to ingest them and so their transportation by this means is out of the question. The diaphragm of a guinea-pig killed fifteen minutes after injection of lampblack shows, as has already been described, the lymphatic vessels marked out in black. Sections at this stage indicate that the polynuclears could have had little, if anything, to do with the transportation of the particles, for the lampblack in the lymphatics is entirely free, none is contained within cells, and it is not until the pigment reaches the anterior mediastinal nodes that any phagocytic action may be observed. The sections also show many particles clinging to the peritoneal side of the diaphragm and also apparently being forced through between the cells of the peritoneal endothelium. In the later stages, however, the wandering cells certainly play an important part in the transportation of lampblack to the lymphatics of the diaphragm. The lymph spaces are now crowded with microxycytes and macrophages filled with large black granules, more particularly the latter. Rarely, too, a wandering cell filled with particles may be seen in the act of crawling into the diaphragm between the endothelial cells of the peritoneal surface. Lampblack is also still passing through in a free condition.

Leaving unsettled the question of the presence or absence of " stomata," it may be said in conclusion that the structure of the peritoneal side of the diaphragm permits of a very rapid passage of inert particles, bacilli, and chicken blood corpuscles from the peritoneal cavity to the mediastinal lymphatic system; and that leucocytes have nothing to do with this " initial rush."

GENERAL CONCLUSIONS.

1. After intraperitoneal injection of suspensions there is an immediate rush of particles to the lymphatics of the diaphragm.

2. From the diaphragm the particles are carried along the anterior mediastinal lymphatic trunks, and through the lymph nodes, reaching the thoracic ducts and blood current almost immediately after injection.

3. Throughout their whole course from the peritoneal cavity to the organs the particles in the earliest stages are practically in a free condition, being taken up later by the phagocytes.

4. In the anterior mediastinal lymph nodes phagocytosis by the macrophages is the most prominent feature, the microxycytes taking comparatively little part. This applies, not only to inert particles and animal cells, but also to bacteria.

5. Whether the particles pass into the lymphatics of the diaphragm by stomata or gain entrance between the endothelial lining cells we have been unable to decide.

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ABSORPTION OF PARTICLES FROM THE PERITONEAL CAVITY.

PART IV. — *The function of the omentum.*

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We have fully discussed (Part III.) the part played by the diaphragm in absorption of particles from the peritoneal cavity, and have shown how the initial rapid rush of particles or bacteria in a free condition to the organs may take place by way of the lymphatics of the diaphragm. It now remains to consider whether there is any other channel by which the particles can leave the peritoneal cavity in a free state.

The omentum appears to furnish such an additional channel to some extent. Sections made from the omentum within an hour after injection of lampblack into the peritoneal cavity show that the afferent plexuses of the lymph nodes are filled with free particles. After two or three hours the particles have been taken up by the macrophages and lie principally in the sinuses of the node.

All this corresponds precisely with processes seen in the anterior mediastinal lymph nodes, so without going into further details we may take it as probable that the lymphatics of the omentum, as well as those of the diaphragm, are concerned in the initial rapid rush of particles to the organs, though probably to a less extent.

That there are any other parts of the peritoneal surfaces which participate in this initial rush seems doubtful. After injection of lampblack both ventral and dorsal abdominal surfaces of the peritoneum can be washed gently under the tap and every trace of pigment disappears from the surface. No black points can be observed, even with the aid of a pocket lens. Ricoux[1] has described minute lymphoid follicles on the abdominal surface of the peritoneum of guinea-pigs. The follicles consist entirely of macrophages which thrust out protoplasmic processes between the lining endothelial cells. The processes englobe particles (of carmine) and then retract, drawing the carmine with them into the follicles, from which point the macrophages, containing the particles, wander into the lymph spaces and channels. In consequence of this process the abdominal wall, shortly after injection of carmine, appears covered with minute pink spots visible to the eye.

With lampblack we have been quite unable to confirm this observation of Ricoux, and even if he is correct in his statements, the process he describes is too slow to account for the rapid rush to the organs. We feel convinced that the abdominal parietes of the peritoneum are not concerned in it.

The same, so far as we have been able to observe, is the case with the mesentery. It can always be washed free of particles in early stages, although later the mesenteric nodes show infiltration of pigment. This infiltration is probably a secondary process, as suggested by Muscatello, the pigment arriving by way of the circulation; and also perhaps to some extent caused by phagocytes which have picked up particles in the peritoneal cavity, and worked their way into the mesenteric tissues.

Unlike the abdominal surfaces or the mesentery, the omentum becomes intensely blackened almost immediately after injection of lampblack, and the pigment cannot be washed off under the tap. The omentum, therefore, appears to play an important part aside from the rapid infiltration of its lymph nodes, already mentioned, since this infiltration alone would not account for its intensely blackened appearance. We find, indeed, that phagocytosis is exceedingly active on the surface of the omentum, and the phagocytes, after picking up particles, find their way into the omental tissues where their morphological characters become changed in a remarkable manner, as we shall see later. But the primary, immediate blackening of the omentum is mainly due to a deposit on its surface of fibrin in which the particles become entangled, and where they await the arrival of the phagocytes. In order to appreciate the details of the phagocytic activities of the omentum it will be advisable to describe its normal histological appearance before attempting to deal with its functions under pathological conditions. Our remarks refer to rabbits and guinea-pigs.

Normal histology of the omentum. — If a piece of the omentum of a young rabbit is spread out over a microscopical slide and held up against the light,* it appears as a semi-transparent sheet dotted over with a number of opaque spots, the "tâches laiteuses" of Ranvier. Blood vessels course in various directions, some of which can be seen entering the milky spots, but others do not reach them. A milky spot is the precursor of a capillary network or tuft, and a large piece of the omentum spread out shows a tracery work of spots, marking out the courses of the future blood vessels (Plate III., 1). In a full grown rabbit the milky spots are all occupied by blood capillaries.

With the guinea-pig the milky spots are not apparent. The capillaries work their way out over the sheet singly and only form tufts exceptionally; the few tufts which may be seen not being preceded by a milky spot without vessels.

Let the omentum spread of the young rabbit be stained with eosinate of methylene blue, and examined under a low power of the microscope. The milky spots stand out as thick masses with the center often stained deeply in blue; some of the spots being already traversed by numerous capillaries. Plate III., 2, shows a milky spot without, and Plate III., 3, a milky spot with capillaries.

Over the surface of the omentum there are sometimes a number of violet dots, though these are not often present. They have no connection with the milky spots, and are more often present in the guinea-pig than in the rabbit.

The omentum can now be examined under a high power of the microscope (1/12 oil immersion lens).

1. The violet dots. — These are seen to be very large cells with a large round or ovoid nucleus, deeply stained in violet, the nucleus being surrounded by more or less scattered large violet granules. The cells appear to be very

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* We shall call this an "omentum spread" for brevity.

unstable, and it is impossible to find any definite limit to the cytoplasm. Even the nucleus is generally broken up into granules.

These are the mast cells of Ehrlich. Westphal says that they do not occur in the rabbit, but we have certainly seen traces of them occasionally. In the guinea-pig they are often frequent, clinging mostly along the long axis of the smaller blood vessels. But they are most conspicuous on the mesentery of the guinea-pig, where they occur as large flat cells, apparently lying just below the surface epithelium (Plate VIII., 2).

2. The milky spots. — (*a*). Without capillaries: If one of the smaller milky spots is examined under a high power, it appears at first sight to be made up of a number of cells with branching processes of the nature of connective tissue cells. The nuclei are round to oval in shape, and from either end of a nucleus there is usually a relatively long process, the other branches being shorter and less conspicuous. There are also a few large round mononuclear cells apparently lying on the surface. Between the large round mononuclear cells and those with long processes, which may provisionally be called "elongated cells," may be seen a certain number of intermediate forms. The picture gives one the impression that the round cells are gradually working their way into the tissue, where they finally appear as the elongated cells. A few, both of the round and elongated cells, may contain small dark or reddish staining granules (Plate VIII., 3). In the center of the larger spots, in addition to the cells described above, there are masses of smaller lymphoid cells, stained deeply in blue. It is these cells which cause the center of the milky spot to appear deep blue under a low magnification.

b. With capillaries: In addition to the above elements, tortuous capillaries now traverse the milky spots in all directions. The lymphoid cells have increased greatly in number and appear to have changed somewhat in character. They now show a rim of deep blue basophile cytoplasm with a relatively pale-staining nucleus (Plate VIII., 1). The large round mononuclears may still be seen in places, and the elongated cells to a great extent have arranged themselves so that they lie parallel with the axis of the capillaries (Plate IV., 3, may be referred to as an example of this arrangement). In this situation they appear with two long processes issuing from either end of the oval nucleus, the side branches being absent or inconspicuous. Occasionally, though very rarely, both large, round, and elongated cells may be seen to contain what is obviously the remains of an eosinophile cell or of a microxycyte. We may suspect, therefore, that in these large round cells we have the macrophages of Metchnikoff, and since we find remains of eosinophiles in the elongated cells also, the surmise already made that these are round cells which have worked their way into the tissue receives some support. It seems probable that the reddish granules previously described as occurring normally in the cells of a milky spot represent a later stage in the digestion of the eosinophiles, while the dark granules may be remains of nuclei or particles of extraneous matter picked up in the peritoneal cavity. We shall find these surmises fully confirmed when we come to deal with the omentum under pathological conditions, and a glance forward may be permitted here (Plate IX., 1), where is a complete history of the process of evolution when there is a large amount of pus in the peritoneal cavity.

3. The connective tissue meshwork. — Immediately around the blood vessels and milky spots the omentum forms a sheet of connective tissue covered on both faces by the endothelium of the peritoneum, but at a short distance on either side of the vessels or milky spots the continuous sheet becomes broken up into a meshwork, the trabeculæ of connective tissue being covered on all their faces by flat epithelial cells with vesicular, pale-staining nuclei. Here and there, also, are to be seen elongated cells with two, three or more branching processes like those of the milky spots, but as a rule more distinctly elongated. Occasionally a large round mononuclear cell or a polynuclear eosinophile cell may be seen on the surface. In the guinea-pig the meshwork

is much more open and the connective tissue of the trabeculæ much denser than in the rabbit.

As has already been remarked, there are no milky spots in the omentum of the guinea-pig, but in the neighborhood of the capillaries and in the meshwork the elongated cells can be found in large numbers. In the connective tissue of the meshwork they are very conspicuous, rarely presenting more than two or perhaps three very long, finely drawn out processes, in which reddish, eosinophile granules are much more in evidence than with the rabbit. We first observed these elongated cells in the guinea-pig and named them "trailers," a term which may be conveniently used to designate them.

4. The trailers (Plate IX., 2). — Although there is at first sight a very marked difference between such trailers as seen in the meshwork of the guinea-pig and the more branching cells of the milky spots of the rabbit, we think that the evidence is in favor of their being the same cells, simply varying in appearance on account of their environment. The tissue of the milky spots appears loose and somewhat of an areolar nature, so that the macrophages on entering it can put out processes in various directions. In the neighborhood of the capillaries or in the meshwork of the rabbit the connective tissue is denser, so we find the cells more distinctly elongated while the side processes are comparatively short and few in number. In the meshwork trabeculæ of the guinea-pig the tissue is much denser still, and here we find the typical trailer with its two, or at most three, very long, finely drawn out processes feeling their way along the narrow channels between the connective tissue bundles.

All these cells, wherever found, give evidence that they are, or have been, of a phagocytic nature; evidence which is more marked under pathological than under normal conditions, as we shall see later. The question whether or not these cells become fixed connective tissue cells will have to be postponed for discussion until we shall have become more familiar with the trailers under pathological conditions.

Ranvier appears to have been the first to describe the milky spots. He refers to them as being made up of connective tissue cells and lymphatic elements. With regard to the lymphatic elements Dominici[2] remarks that there are cells in the omentum of the rabbit comparable with lymphocytes. They predominate in the milky spots, and undergo various transformations. Some model themselves into endothelial, vasoformative or fat cells and incorporate themselves with such structures, while others preserve their rounded form, increase in size, and become macrophages. In his opinion the connective tissue cells can become mobile and change into cells identical with macrophages, and again the macrophages can become fixed connective tissue cells. Dominici's views are opposed to those of Retterer,[3] who maintains that the omentum develops as a double sheet of epithelium, and from the epithelial cells are derived the connective tissue cells and meshwork. The lymphoid cells of the milky spots also develop from the epithelium.

Jolly[4] considers the lymphoid cells as identical with the plasma cells of Unna. Opinions are divided, therefore, as to the origin of the lymphoid cells of the milky spots, and we may content ourselves with the fact that they exist in the forms already described. We shall not find them of any particular importance until we come to deal with immunized animals in a future series of papers.

When we first began examining the omentums of animals after infection of various substances, the observations were only intended as an adjunct to the work treated of in the three previous articles. It was only by degrees that we came to realize the full significance of the processes which take place on the omentum, and we had already worked out all that has been said by us on the subject of the trailers before investigating the literature on the subject, when we found that our observations had been anticipated.

We were under the impression that Ranvier's clasmatocytes were identical with Ehrlich's mast cells, and it was not until Ranvier's[5] articles came into our hands that we discovered the clasmatocytes to be trailers under an older and more scientific name.

Ranvier at first in a series of articles in the Comptes rendues Academie des Sciences, 1890 and 1891, described the clasmatocytes as occurring in various situations in amphibia and mammals; in the latter being especially noticeable on the omentum of rats, rabbits, and guinea-pigs. He described them as being large mononuclear leucocytes which had wandered into the connective tissue and there, becoming fixed, secreted the granules which are such a marked feature in their cytoplasm. After a time the cytoplasm of the cells bursts, liberating the granules which are scattered free in the surrounding connective tissue — clasmatosis.

Later he injected carmine into guinea-pigs intraperitoneally, and after twenty-four hours found the carmine granules in the clasmatocytes. He suggests that the particles of carmine have been taken up by the large mononuclear cells which. have then wandered into the connective tissue and become clasmatocytes. This is precisely our opinion, but Ranvier does not seem to have realized that the granules seen in the trailers of normal animals are probably granules derived from polynuclear cells and extraneous substances which have been ingested. We think that we have clear evidence that this is the case.

Renaut[6] in addition to the clasmatocytes on the omentum of the rabbit finds in the milky spots other somewhat similar cells containing minute acidophile granules. Such cells branch much more freely than the clasmatocytes and have a much greater resemblance to ordinary connective tissue cells. These cells he calls rhagiocrines (ῥαγιον — a small grape seed) and he considers them as a variety of connective tissue cell which secretes granules, whereas the ordinary connective tissue cells do not, their cytoplasm being homogeneous. The rhagiocrines are derived from the large round cells of the peritoneum. In a later communication[7] he describes the rhagiocrines as of a phagocytic nature, since twenty-four hours after injection of lycopodium powder the rhagiocrines are found to contain particles of the powder. Renaut's rhagiocrines are obviously identical with the cells of the milky spots previously described by us (figured on Plate VIII., 3). We have already ventured our opinion that these cells cannot be differentiated from the " clasmatocytes " of Ranvier or our " trailers." Both are derived from macrophages which work their way into the tissues and there present somewhat different appearances according to the nature of the tissue.

Marchand[8] is of the opinion that the clasmatocytes are a variety of connective tissue cells which have no connection with macrophages, but possess the property of becoming phagocytic under inflammatory conditions. The clasmatocytes occur chiefly in the adventitia of the blood vessels.

The cells of the peritoneal fluid. — We have already touched on this subject in Part I., but we may summarize again, since these cells are of importance when studied in connection with the omentum. Kanthack and Hardy have established the presence of small and large lymphocytes in the peritoneal fluid, and eosinophile leucocytes. Amphophile polynuclears, or the microxycytes of Durham, are normally absent or few in number. The large lymphocytes (hyaline cells) are abundant. They possess a large vesicular nucleus and homogeneous, slightly basophile cytoplasm.

The large round mononuclear cells previously described as occurring on the surface of the omentum we take to be identical with Kanthack and Hardy's hyaline cells, and we have already indicated the important part they play as phagocytes. Eosinophile leucocytes are also abundant, and it is no doubt due to this fact that so many can be found on the surface of an omentum spread.

The omentum under pathological conditions. — The omentum of a guinea-pig, as already mentioned, is blackened almost immediately, even in five minutes, after injection of lampblack intraperitoneally, and remains pigmented for months. Three months is the limit of our observations. It is then still black. Shortly after injection the guinea-pig is killed and a piece of the omentum spread out on a slide. In many places the appearance presented by the photograph

(Plate IV., 1) of an unstained specimen immediately strikes the eye. The lampblack is not scattered diffusely over the surface but appears as a network of irregular black lines. The same may be observed in a stained specimen after injection of typhoid or other bacilli into a rabbit or guinea-pig.

On focussing down on these lines with a one-twelfth oil immersion lens the particles of lampblack or bacilli are found to be lying free in what appear to be narrow channels. The particles are not enclosed in phagocytes, and in the case of the bacilli they are all pointing along the long axis of the channel as if passing along in a current. The photograph (Plate V., 1) is one of typhoid bacilli thirty minutes after injection into a rabbit, and Plate V., 2, coli communis fifteen minutes after injection into a guinea-pig.

At first it was thought that these apparent channels must be the swollen lymphatics of the omentum into which the particles or bacteria had forced their way, much as they appear to do in the diaphragm, but this was soon found to be a mistaken idea. The strands of the network are simply fibrin which has been deposited on the surface of the omentum and has caught up the particles or bacilli in it. There were several reasons for suspecting that these apparent channels are not in reality lymphatics. In the first place they often seem to run a little way and then suddenly come to an end. Again, the strands run quite independently of the omental meshwork, nor could any nuclei be discovered in them. Their real nature was finally determined by means of Weigert's stain for fibrin, to which they unmistakably responded. The photograph (Plate IV., 2) was taken from the omentum of a rabbit two hours after injection of typhoid bacilli where the strands of fibrin were numerous, but contained few bacilli. The amount of fibrinous deposit varies very much. It may be extensive but often is quite inconspicuous.

We have dwelt somewhat at length on this subject since we have been unable to find in the literature any clear description of the appearance of the fibrin. Pierallini, who was referred to in Part I., merely speaks of masses of fibrin, though Ranvier[9] in a short communication mentions that after injection of silver nitrate into guinea-pigs there is a deposit of fibrin like a spider's web on the surface of the omentum. Ranvier, 1896, also says that the lymphatics of the omentum follow the blood vessels but do not extend into the meshwork. Since abandoning the idea that the network described above represents the swollen lymphatics, we have been unable to find the lymphatic channels by which the omental nodes, found in sections to be quickly blackened, are supplied. However, apart from the question of the initial rush of particles to the organs, the omentum plays a very prominent part in the local phagocytic processes of the peritoneal cavity, and this phase of the subject will now be fully discussed.

To follow the argument it will be necessary to refer briefly to the processes in the fluid of the abdominal cavity. We have already described how with lampblack, after a period of leucopenia, the microphages (microxycytes) first come in, and later the macrophages. The same occurs also after injections of bacteria, but with them we have not found it so easy to follow the processes in detail.

I. THE OMENTUM IN EARLY STAGES AFTER INJECTION.

a. Chicken red cells. Early stages.—We may first examine the omentum spread of a guinea-pig which has been injected a few hours (4–8) previously with chicken red cells. We find on the surface and in the meshes an enormous number of large, round macrophages, singly or in masses, stuffed full of chicken cells and some containing also polynuclear leucocytes, more or less degenerated, many of the macrophages clinging to the strands of fibrin (Plate X., 1).

Not only are the macrophages a prominent feature, but a great many of the trailers may now contain relatively large, dark blue, almost black staining granules as well as the usual small, black and red granules which we have already described in the normal omentum. Some of the trailers may contain only dark blue granules. One cannot help suspecting that the macrophages have collected over the surface of the omentum with the object of penetrating into the tissues, but are unable to do so until they have so far digested the chicken cells that they can carry the remains in with them, or in other words, until only the nucleus of the chicken cell is left.

Once the macrophages have entered the meshwork they trail along the narrow spaces between the connective tissue bundles. Occasionally, though very rarely, indeed, one may find a trailer containing the remains of one or two chicken red cells still retaining their cytoplasm (as figured in Plate X., 1, on left), but such an appearance is so very uncommon that one would hardly be justified in concluding that the cells are actually macrophages, which after englobing red cells have become trailers, any more than we could be certain about this with the trailers of the normal animal.

The colored drawing (Plate X., 1) is taken from an omentum spread five hours after injection of chicken cells, but trailers containing nuclei and more rarely one or two degenerated chicken cells may already be seen in one hour after injection. One hour, however, is the earliest period at which such trailers can be found. In fifteen and thirty minutes there is generally a considerable deposit of fibrin in which are entangled masses of chicken cells and hyaline cells of the peritoneal fluid, but the latter have only to a slight extent begun to englobe the chicken cells. After one hour, however, phagocytosis by the macrophages is very marked.

b. Lampblack. Early stages. — The appearances on the omentum are somewhat different from those with chicken cells. We find almost immediately after inoculation the usual deposit of fibrin in which are entangled lampblack particles and hyaline cells of the peritoneal fluid (macrophages), but the latter are already packed with black particles. Indeed, they are packed so full that they appear to be unable to enter the tissues, and it is not until six hours after injection that trailers begin to make their appearance. At this period, however, we find our surmises as to the origin of the trailers from macrophages fully confirmed. Lampblack being indigestible undergoes no change in the cells, and there can be no question whatever that the trailers actually contain particles of the pigment which cannot possibly be mistaken for anything else. We find also a number of pigment-laden intermediate forms between the round macrophages and the elongated trailers. The macrophages appear to send out protoplasmic processes which insinuate themselves between the lining endothelial cells and penetrate into the connective tissue meshwork, dragging the particles with them (Plate X., 2).

We mentioned in Part I. that the macrophages appear to englobe chicken cells with much greater avidity than particles of lampblack. From our earlier observations on the peritoneal fluid we supposed this to be the case and have left the paragraph there as it was originally written several months ago. Our studies of the omentum, however, have gradually led us to consider that the greater avidity for chicken cells is only apparent. A macrophage which has englobed a dozen chicken cells presents a very remarkable appearance, whereas a similar number of small lampblack particles in a macrophage is not very conspicuous. Moreover, when free in the peritoneal fluid the macrophages are to some extent at a disadvantage, and small particles are probably more elusive than large ones. It is a well-established fact that ameboid cells can englobe particles much more readily if they are clinging to a surface over which they can crawl than when they float free in a fluid. The surface of the omentum, therefore, affords a better idea of the capabilities of the macrophages than the peritoneal fluid. Indeed, the macrophages entangled in the fibrin have a double advantage over those free in the fluid. Not only are they themselves supported, but they are in close proximity to particles which are fixed in the fibrin and not floating free.

This affords a clue as to why phagocytosis of lampblack takes place so much more rapidly than that of chicken cells in this situation. The particles of lampblack are so small that the macrophages can pick them up quickly, one by one, until they are stuffed full in a very short time. To ingest a large chicken cell a great deal more work has to be performed, so it is distinctly longer before the macrophages show much evidence of their activity with such cells. Once the chicken cells have been taken up, however, the macrophages digest them very speedily, and it is not long before the load is lightened and the macrophages are able to penetrate into the tissues and from trailers, whereas the lampblack particles remaining unchanged delay the entrance. In fact, we are inclined to suppose that the primary overloaded hyaline cells do not enter the tissue at all, but that the trailers seen in six hours after inoculation of lampblack are derived from a fresh supply of macrophages which are not overloaded like the primary ones (an overloaded macrophage is represented in Plate V., 4).

II. THE OMENTUM IN LATER STAGES AFTER INJECTION.

a. Chicken cells. — In twenty-four hours the picture has changed very considerably. The large masses of macrophages stuffed with chicken cells are no longer a prominent feature. A great number of macrophages may still be seen, but the chicken cells contained in them have greatly degenerated, and little is left but the nuclei. On the other hand, trailers are very abundant, and many of them contain dark blue granules, no longer recognizable as nuclei of chicken cells, but probably representing their fragments. The majority of the round macrophages which are still left on the surface show signs of degeneration. They stain badly and are often riddled with vacuoles, the vacuoles probably representing the localities of the digested chicken cells. It appears probable that such cells will not enter the tissues, but have perished after performing their work of digestion.

In forty-eight hours the omentum has almost resumed its normal appearance, but few macrophages can be seen; some contain remains of chicken cells and are apparently dead, but many, which for the most part stain readily, show no traces of them, and are probably late comers for whom no extra work of a pathological nature remains to be done. The chicken cells have all practically been disposed of. It is naturally understood that the rapidity of the process depends to a great extent upon the amount of the dose injected. If a large dose has been given, the destruction of the chicken cells occupies a longer period, but the final result is the same.

The remarkable similarity of the processes on the omentum with those in the anterior mediastinal lymph nodes, previously discussed, may be noted.

b. Lampblack. Later stages. — Since lampblack is indigestible, the later appearances are very different from those met with in the case of chicken cells. In twenty-four hours and forty-eight hours we still find the round macrophages loaded with pigment on the surface in large quantities, but there is distinctly an increase in the number of trailers containing black particles. Microxycytes are still numerous in twenty-four hours, and a large proportion contain pigment, but in forty-eight hours show a considerable decrease.

As has been remarked, the omentum remains black to the eye for a long time, and under the microscope, even one month after injection, we still find on the surface the masses of round macrophages packed with pigment, but they appear to have migrated largely to the neighborhood of the capillaries and small vessels. Or possibly newly-formed capillaries may have made their way to the macrophages and there ramified through the masses. But we are inclined to think the former view the more probable (Plate IV., 5).

At this stage — one month — however, the trailers have changed their characteristics to a great extent. Most of them still contain pigment, but the particles are for the most part now distinctly lying in vacuoles, and have undergone fragmentation, so that each vacuole contains a great number of exceedingly minute black granules.

Plate IX., 3, shows two trailers one month after injection of lampblack. The cells contain large vacuoles in which are minute fragmented particles. The photographs (Plate

IV., 3 and 4) were also taken from a preparation at this stage. No. 3 shows very well the manner in which the trailers tend to range themselves along the long axis of the capillaries and small vessels, and No. 4 represents a trailer with a vacuole in which the particles are partially fragmented.

In many cases vacuoles may be seen which are nearly or quite empty. This probably represents the "clasmatosis" of Ranvier — the bursting open of the cytoplasm and liberation of the granules — see page 62. It is interesting to note also that at this stage many very minute free granules may often be seen, although we do not feel certain that they are particles scattered in consequence of clasmatosis. It seems probable that the processes of fragmentation and clasmatosis here described represent an effort on the part of the organism to rid itself of the particles. Being now in such a minute state of division they would be more readily excreted by various channels than when in their original condition.

The polynuclear leucocytes. — It remains only to say a few words about the polynuclear leucocytes. In the earlier stages practically no microxycytes are to be seen, although there may be a considerable number of eosinophile cells on the surface of the omentum, many of them entangled in the fibrin. The eosinophiles do not appear to take any prominent part in the processes described, although rarely they may be seen to contain a few particles of lampblack. This is in accord with the opinions of most authors who seem to be generally agreed that eosinophiles are only feebly, if at all, phagocytic. In Plate X., 2, an eosinophile containing particles of lampblack is figured, but this is an exceptional case. Throughout the whole period covered by our observations a few eosinophiles may be seen in the omentum. They do not appear either to increase or to decrease in number and the part they play is, so far as can be judged, a purely passive one.

Microxycytes, however, arrive in vast numbers, beginning to appear in about one hour after inoculation, and increasing in number up to eight hours after injection of chicken cells. In sixteen hours they are already less numerous, and in forty-eight hours are scarcely to be found. Probably they reach their maximum in about twelve hours, but we have not bridged the gap between eight and sixteen hours.

After injection of lampblack the microxycytes persist much longer than after chicken cells, being still very numerous in twenty-four hours, although in forty-eight hours there are comparatively few. We are inclined to suppose that the microxycyte reaction is as great in one case as in the other, but lampblack being indigestible, the macrophages are not able to turn their attention to the microxycytes and englobe them so early as in the case of chicken cells. During the first few hours microxycytes may be seen streaming out from the small vessels and capillaries in vast numbers, often followed by masses of red cells, so that there is a distinct though slight hemorrhage following the track of the vessels. The microxycytes do not ingest entire chicken cells, although occasionally one or two nuclei may be seen inside them. Nor have we observed on the omentum appearance mentioned in Part I. of a microxycyte forming a center around which is grouped a mass of chicken cells with partially eroded cytoplasm. But free chicken cells are rarely seen on the surface of the omentum after the first hour. By the time the microxycytes arrive all the chicken cells entangled in the fibrin have been englobed by the macrophages.

The microxycytes, however, take up lampblack readily, and between four and eight hours after injection a large proportion of them contain particles. But they do not gorge themselves as do the macrophages. After taking up a few particles their activity seems to become exhausted and they then, so far as can be judged, remain passively inert, waiting to be taken up, particles and all, by the macrophages, so soon as the latter arrive in sufficient quantity. If there is any other physiological reason for their existence besides that of acting as assistants of the macrophages, it is not apparent from an examination of the omentum spreads.

CONCLUSIONS.

1. Almost immediately after injection of inert particles into the peritoneal cavity of a guinea-pig there is a deposit of fibrin formed on the surface of the omentum in which the particles and phagocytic cells of the cavity become entangled.

2. The phagocytic cells (macrophages) rapidly englobe the particles, becoming filled with small particles like lampblack within ten minutes, and with chicken red cells in about an hour.

3. If not overloaded the macrophages then enter the tissues and appear as long trailers or clasmatocytes.

4. If the particles are digestible, *e.g.*, chicken red cells, they are rapidly disposed of by the phagocytes.

5. If the particles are not digestible, *e.g.*, lampblack, they remain inside the macrophages and trailers for months. The particles in the trailers become fragmented, and after a time are discharged as minute fragments (clasmatosis of Ranvier).

6. The macrophages are the most active agents of phagocytosis on the omentum, the polynuclear leucocytes playing a minor part.

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ABSORPTION OF PARTICLES FROM THE PERITONEAL CAVITY.

PART V. — *The function of the omentum, continued.*

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For the experiments detailed in the previous section guinea-pigs were used, but in making parallel tests with typhoid bacilli, omentums of rabbits, the organs of which were studied in Part II., have been employed. The omentums of these rabbits, with the exception of a few of the earlier ones, were cut out and dropped into one per cent formalin, a spread being made about two hours later, after the plating out of the organs was finished. The spreads were dried and stained with eosinate of methylene blue. No special fixation is necessary, and the stained specimen can be mounted in Canada balsam.

The processes on the omentum of a rabbit which has been inoculated with bacilli are somewhat more complicated than with the guinea-pig, on account of the presence of the milky spots (Plate III., 1, 2, 3) in which Renaut's rhagiocrines, our modified trailers (depicted in Plate VIII., 3), are to be found in great abundance. These modified branching trailers often appear to become filled with bacilli almost immediately after injection of typhoid, and one of the objects of this study has been to attempt to determine if the original branching cells of the milky spots are actually of a phagocytic nature, or if those which are seen to contain bacilli after inoculation are newly formed branching cells derived from macrophages which have englobed bacilli and become quickly transformed. In addition to this there are many other interesting points which arise in the course of the study and these will also be discussed in detail.

The processes in the fibrinous deposit. Early stages. — Shortly after injection of typhoid bacilli there is the usual deposit of fibrin on the surface of the omentum, in which may be entangled large masses of bacilli pointing chiefly along the long axis of the strands. This appearance has already been commented upon (page 64, Plate V., 1 and 2). If, according to the evidence on the plates, the bacilli appear to have been destroyed explosively (page 30), none can be seen entangled in the fibrin, and the deposit of fibrin itself is usually scanty. We shall return to this aspect of the phenomena later on, confining ourselves for the present to the first mentioned instances in which intact bacilli are entangled in the fibrin.

The bacilli at first lie free in the fibrinous deposit, but by degrees are taken up by the macrophages entangled alongside of them. This englobement is rarely complete in the earlier stages, the bacilli being far too numerous for the original macrophages to deal with. The macrophages already present, however, very quickly become filled with bacilli, and a single one may often contain fifty or more within half an hour after injection.

One of two things may now happen:

1. The bacilli may become pale staining or granular. Corresponds to few in the organs.

2. The bacilli may remain intact and increase in the fibrin. Corresponds to many in the organs.

The specimens naturally vary greatly, but in omentum spreads four to six hours after inoculation one or the other of these phenomena will be in the ascendant.

1. The bacilli become pale staining or granular. — In this case the bacilli will be found in the interior of the macrophages and perhaps also to some extent in microxycytes, which by this time will have arrived in considerable numbers. Probably no free bacilli will be found in the fibrin, and those in the cells appear either pale staining and somewhat swollen, or else as small, deep blue-staining granules, representing shrunken and distorted bacilli.

In the wash water and organs are few or no bacilli.

2. The bacilli remain intact and increase in the fibrin. — The main feature after four to six hours is the numerous, roughly spherical, packed masses of bacilli (Plate V., 3).

Some of the groups appear to be agglutination centers, in which the bacilli are now growing freely. Others appear to have started in the interior of macrophages, or occasionally in microxycytes; the phagocytes having probably taken up more bacilli than they could dispose of. Such cells often present a very remarkable appearance, being literally packed with vigorous bacilli, which stain perfectly and are evidently rapidly multiplying. When the phenomenon of packed masses of bacilli is predominant, there is usually relatively little microxycyte reaction.

In the wash water and organs bacilli are numerous.

It was noted in Part II. that rabbits killed at four and six hours after inoculation show much higher averages of living bacilli in the peritoneal cavity than those killed in one and two hours. The phenomena just detailed appear to support the correctness of the earlier observations. After inoculation there is probably an immediate reaction on the part of the animal, during which the greater part of the bacilli are destroyed, either explosively or during the first hour or two. In many cases, however, the reactive power becomes exhausted and those bacilli which remain alive are able to multiply again, not only when free in the peritoneal cavity, but also on the surface of the omentum, even though they have been taken up by the phagocytes.

The processes in the fibrinous deposit. Later stages. — Much the same condition as for four to six hours may hold good up to sixteen hours; the main feature may be granule formation or increase of bacilli in the fibrin. From what has been observed to occur it seems certain that those rabbits in which secondary multiplication of bacilli persists up to sixteen or twenty-four hours would succumb. In fact, on the omentum of one rabbit, which died twenty-three hours after inoculation (Part II., Table XI., 6), the phenomena of packed macrophages and dense masses of bacilli in the fibrin were present to an exaggerated degree. There was little microxycyte reaction.

The fibrin after sixteen and twenty-four hours in most cases is swollen, with indistinct outlines, and is surrounded by microxycytes which appear to be attacking it (Plate V., 5).

In forty-eight hours only traces of fibrin, packed with microxycytes, are left. If any bacilli can be seen at all they are pale or granular. It may be noted that rabbits killed forty-eight hours after inoculation are always on the highroad toward recovery. If inoculation is followed by death it always occurs within twenty-four hours.

Phagocytosis on the meshwork. — The foregoing descriptions refer to the processes as seen in the fibrinous deposit. Scattered over the meshwork will also be found a few macrophages, intermediate forms and trailers, which contain bacilli in all stages from intact to pale staining and granular. The condition of the bacilli contained in the phagocytes corresponds in each specimen with that in the phagocytes of the milky spots, in the account of which further description of the processes on the meshwork will be included.

Phagocytosis on the milky spots. Early stages. — The bacilli appear to be caught up and fixed over the milky spots in enormous numbers, and it is here that phagocytosis is most pronounced. In the macrophages and branching cells may be found bacilli, either intact or granular, the appearances in each particular instance corresponding very closely with those observed in connection with the fibrin. If granules predominate in the phagocytes of the fibrin they will predominate also in the cells of the milky spots, and the same applies if the bacilli remain intact.

We may now consider a typical case in which the rabbit was bled from the carotid immediately after inoculation (Table IX., 1, in Part II.) and the omentum removed as quickly as possible after the peritoneal cavity had been washed out. The rabbit was dead in ten minutes after inoculation and not over ten minutes more had elapsed before the omentum was taken out. The wash water contained many millions, and the organs were swarming with bacilli.

There is not much deposit of fibrin at such an early stage, but over the milky spots we find immense masses of intact bacilli, which stain well and give no evidence of granular degeneration. Closer examination shows that the bacilli are for the most part already enclosed in cells. Not only do the round macrophages on the surface of the milky spots contain enormous numbers, but a large proportion of the branching cells — the modified trailers — are also in the same condition. It is difficult to suppose that the macrophages have enclosed so many bacilli and transformed themselves to such a marked extent into branching cells in this short space of time. It appears more probable that the branching cells of the milky spots are themselves of a phagocytic nature. This supposition would lead us to infer that these cells are only partially fixed or anchored in the milky spots, from which position they send out processes, which, waving free in the peritoneal cavity, pick up bacilli floating in the fluid. There is no direct proof that this is actually the case, but it seems to be the best explanation of the phenomena observed.

It is true, however, that macrophages can transform themselves into trailers very quickly. This becomes evident on inspection of the meshwork, where even at this early stage transitional forms may be seen. The photograph (Plate VI., 3) shows early transitional forms, but many cells may be found in a much more advanced stage, and even a few typical trailers containing intact bacilli. Plate XI., 3, is a composite picture from the same specimen taken from three or four different fields of the microscope.

The transformation into trailers is effected much more rapidly when the macrophages contain bacilli than when they are loaded with unyielding masses of lampblack. It was observed in Part IV. that trailers containing lampblack do not appear in the meshwork earlier than six hours after inoculation into a guinea-pig.

The foregoing descriptions are typical of appearances on the omentum in any instance where large quantities of bacilli are found "at once" in the wash water and organs.

We may now consider instances in which the bacilli are destroyed explosively, as recorded in Table IX., 5, 6, 7. No intact bacilli are to be found over the milky spots or elsewhere. Both macrophages and branching cells of the milky spots, however, may contain great numbers of ragged red and black granules with ill-defined margins and varying greatly in size. Such granules it is impossible to recognize as bacilli, but they undoubtedly represent remains of bacilli, since such a picture is not seen with the normal animal in the cells of which the granules are scanty and well defined. This rapid destruction may appear to have mainly taken place either extracellularly or intracellularly.

Extracts from notes of two cases tabulated in Part II. may be cited.

1. Table IX., 5.—No free bacilli or granules. Macrophages and cells of milky spots crowded with reddish indefinite granules (Plate XI., 2).

Wash water, 350,000. Organs, 0.

2. Table IX., 7.—Immense numbers of free bacilli on surface of milky spots and meshwork. The bacilli are shrunken and degenerated. Little evidence of phagocytosis (Plate XI., 1).

Wash water, 1,000. Organs, 0.

Unquestionably in the second instance the bacilli have been destroyed extracellularly, and it seems probable that this also occurred in the first case, the bacilli being then rapidly englobed and transformed into granules inside the cells.

Plate XI., 1.—Shows very well the way in which free bacilli are sometimes caught up in the meshwork lining the open spaces. The form of degeneration in this case is somewhat unusual, and probably represents a stage between the pale-staining, swollen bacilli, and the deep blue, small granules. The basophile substance seems to be massed in the center of each bacillus, leaving a pale-staining sheath.

Phagocytosis on the milky spots and meshwork. Later stages.—The bacilli caught up in the milky spots appear in later stages to undergo much the same fate as already described for those in the fibrin. In four and six hours there may be a decided microxycyte reaction, and in such cases the phagocytic cells may contain pale-staining bacilli or granules. Sometimes, however, practically no bacilli or granules can be found at all. This condition probably represents an effective initial destruction of the bacilli. The pale-staining bacilli or granules observed in analogous cases, "at once" (*e.g.*, Table IX., 5, 6, 7), having been cleared off by this time.

The following are typical instances:

Four hours, Table II., 6. — Microxycytes plentiful. A few macrophages and trailers containing small granules. No intact bacilli.

Wash water, 4,000. Organs, 0.

Six hours, Table III., 6. — Microxycytes very numerous. No small granules or bacilli, either free or in phagocytes.

Wash water, 0. Organs, 0.

Generally, however, at four and six hours the microxycyte reaction is not yet very pronounced, and in such cases, although most of the phagocytic cells contain pale-staining bacilli or granules, there are sure to be some macrophages which are becoming packed with growing bacilli, and also probably a few masses of agglutination centers. It is not often that the animal is able entirely to prevent secondary multiplication. The secondary multiplication, however, does not often last long, for, in the rabbits killed sixteen hours after inoculation, it was evident in only one case out of five. In four cases there was a good microxycyte reaction, but in the fifth little reaction, while centers of multiplication were numerous and far advanced. The temperature, moreover, had fallen from 102° to 99°, and this rabbit (Table IV., 1) would undoubtedly have died in a few hours.*

Of six omentums examined twenty-four hours after inoculation, not one showed any centers of multiplication. A few pale-staining bacilli and granules might be found in some specimens, but in all of them there were swarms of microxycytes. All of these rabbits would certainly have lived. It has already been mentioned that in one rabbit which died in twenty-four hours centers of multiplication were numerous. After forty-eight hours bacilli or granules can rarely be found at all, and there is generally a considerable decrease in the number of microxycytes. The rabbit has overcome the infection and has practically recovered. Nevertheless, the surface of the omentum usually shows marked changes which may be considered under the next heading.

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* The temperatures of our normal rabbits varied from 100° to 103°. If, after inoculation, there was a fall below 100° which persisted, the rabbit died.

The endothelial and connective tissue cells of the omentum. — The endothelial lining cells normally possess large, pale-staining, vesicular nuclei. During the first hours succeeding inoculation the cells undergo little or no change, but in sixteen and twenty-four hours the nuclei are usually much swollen and often project into the meshes (Plate VI., 4). Durham considers that the swollen endothelial cells peel off from the surface and become phagocytic — equivalent, therefore, to macrophages. We have never seen any evidence that this is the case, and are strongly of the opinion that the endothelial cells of the peritoneum have no phagocytic attributes whatsoever. Ricoux* mentions that he has never found grains of carmine in the interior of endothelial cells of the peritoneum.

In forty-eight hours after injection of chicken red cells into a guinea-pig the omentum, as already remarked (page 68), has almost resumed its normal appearance, and the same may be said after the injection of lampblack, although with the latter macrophages and trailers containing pigment are abundant.

Typhoid bacilli, however, in forty-eight hours after injection into a rabbit show evidence of having caused much more serious inflammatory processes on the omentum. Microxycytes and macrophages have decreased in number, but the omentum is generally much thickened, the meshwork being entirely obscured, or very nearly so. The connective tissue appears edematous, and perhaps increased in amount, and is traversed in all directions by newly formed capillaries. The endothelial nuclei are very indistinct, and may not be distinguishable at all. In the connective tissue are very large numbers of branching, apparently connective tissue formative cells, far more numerous than in the normal animal.

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* Thèse de Paris, 1898, p. 31.

Ranvier observed similar phenomena as occurring after injections of silver nitrate and considered that the endothelial cells transform themselves into connective tissue cells. We have never found any evidence that this is the case, and, knowing that the macrophages containing granules of any kind can enter the tissues and become trailers, it is rather natural to infer that macrophages without granules may enter also and become branching formative cells.

Relations of the macrophages, trailers, and connective tissue cells. — We do not feel at all certain that the macrophages, once they have entered the tissue and become trailers, can be sharply differentiated from ordinary connective tissue cells. If the trailers continue their ameboid movements after having entered the tissue and gradually carry off particles to the spleen or lymph nodes, where they resume their existence as macrophages, they must do so very slowly, since in one or two months after injection of lampblack the trailers show evidence of having existed as such for a considerable time. The fragmentation of the particles and the clasmatosis, commented upon on page 69, point to this conclusion.

It may be, then, that the very numerous branching cells found forty-eight hours after injection represent formative cells derived from macrophages which have not picked up anything in the shape of granules from the peritoneal fluid, or have already disposed of such granules.

To summarize this point: we have found no evidence that endothelial cells and macrophages are interchangeable, but it is by no means impossible that macrophages, trailers, and connective tissue cells are all one and the same cell under different conditions of environment, acting as scavengers or formative cells, according to the needs of the organism.

A few observations bearing on this subject may be quoted from the literature.

Dominici (Arch. de Med. Exp., Vol. 14, 1902, p. 20) concluded from studies of the omentum under mild inflammatory conditions that macrophages and connective tissue cells are identical. He agrees, however, with Ranvier that the endothelial lining cells of the omentum can transform themselves into connective tissue cells.

Maximow (Ziegler's Beiträge, Vol. 35, 1904, p. 93) provoked inflammation of connective tissue in rabbits and white rats, and decided that the chief part of regeneration is due to proliferation of preëxisting fibroblasts, but the possibility of mononuclear wandering cells also transforming themselves into fibroblasts cannot be excluded.

Borrel (Annales Pasteur, Vol. 7, 1893, p. 593) induced tuberculosis of the lung by means of intravenous injection and, from a study of the lesions at various intervals, arrived at the conclusion that after primary invasion by polynuclears the tubercles are formed by macrophages, which, on their first arrival, pick up the polynuclear cells, and after digesting these, transform themselves into the spindle-shaped epithelioid cells.

The part played by the microxycytes. — We have remarked in Part IV. that the microxycytes do not englobe chicken cells, but will take up a little lampblack, indigo or other small particles, and then appear to remain passive until they deliver themselves up, particles and all, to be englobed by the macrophages. This process is not so readily followed with typhoid bacilli. Englobement of bacilli or granules by microxycytes is not always to be seen on the surface of the omentum, but from what has already been said, it has become clear that if microxycytes do not arrive in great numbers within four to six hours after injection, the macrophages which have already taken up living bacilli seem incapable of digesting them effectively, and centers of secondary multiplication become more or less manifest. It appears from a close study of large numbers of omentum spreads that the microxycytes themselves are of minor importance as phagocytes in the disposal of typhoid bacilli, but in some way or other assist the macrophages in their work of destroying living bacilli, possibly by virtue of some secretion.

This property of the microxycytes may not apply so forcibly in cases where the granules are formed very quickly after inoculation, although even in such specimens a marked influx of microxycytes is always to be seen.

Whenever microxycytes, after four to six hours, have arrived in great force, the granules always greatly predominate over intact bacilli. Sometimes intact bacilli cannot be found at all, and even the granules are comparatively scanty. In such cases englobement of the microxycytes by the macrophages will be found to have commenced; a feature which becomes much more conspicuous in sixteen, twenty-four, and forty-eight hours, when all stages from the round macrophages to the typical trailer, containing one to four or five microxycytes, may often be traceable within a very few fields of the microscope. Plate IX., 1, showing transitional forms, was taken from a specimen sixteen hours after inoculation.

A frequently noticeable feature in spreads made in sixteen to twenty-four hours is an attack upon the fibrin strands by the microxycytes. Plate V, 5, shows this very clearly, the fibrin appearing swollen with irregular outlines. Clinging to it are swarms of microxycytes, whose business appears to be solely to destroy the fibrin, since the bacilli and granules had already entirely disappeared in this particular specimen.

The granules. — That typhoid bacilli after injection into the peritoneal cavity do not so readily become granular, as is the case with cholera bacilli, is generally recognized, and it is not necessary to refer to the numerous observations which have been reported. Nevertheless, as we have already learned, on the omentum a change of this nature may often be observed. The bacilli at first preserve their natural form, but take the blue stain slightly, and appear pale and often somewhat swollen. They then seem to shrink into small, deeply-staining granules, which later lose their basic properties and are found as somewhat indefinite reddish masses, chiefly inside the trailers and cells of the milky spots. The transformations occur generally within the phagocytes, but degeneration of the bacilli can sometimes be seen to have occurred extracellularly. This is often evident in the earliest stages after inoculation when there has been explosive destruction, and has already been commented upon.

In addition to the small granules which are obviously degeneration forms of the bacilli, since all intermediate forms can be traced, other very large, almost black granules may occasionally be found in enormous numbers contained within macrophages or trailers (Plate VI., 1, 2). It was at first supposed that these granules represent a peculiar form of degeneration of typhoid bacilli, but this was a mistake, since they have also been observed in one instance on the omentum of a rabbit which had received no injection and was intended as a control. The liver of this rabbit presented an extreme case of coccidiosis, and in all of the four specimens where such granules have been a prominent feature, there has been some lesion of the liver observed. We are of the opinion that the large black granules indicate some previous pathological lesions, débris from which has found its way into the peritoneal cavity.

Leucopenia. — The question of the leucopenia occurring in the peritoneal fluid shortly after injections was touched upon in Part I., and may be considered here in relation to the appearances presented by the omentum.

Opinions are divided as to whether leucopenia is due to actual destruction of cells (Metchnikoff) or to a clumping of the cells and their deposition on the omentum or other peritoneal surfaces (Durham). We have never during our studies of the omentum met with any evidence that the hyaline cells or the eosinophiles — that is to say, the principal cells of the normal peritoneal fluid — degenerate or break up. On the contrary, immediately after injections, we find both kinds of cells entangled in enormous numbers in the fibrin, and they can also be observed scattered freely over the meshwork, and over the milky spots.

All these cells, wherever found, appear perfectly normal, and the hyaline cells (macrophages) are obviously in an exceedingly active condition. The cells appear to be simply deposited on the omentum; whether they are swept to it mechanically, or exercise some selective power of their own, it is impossible to say.

Before concluding, a few more words may be said about the milky spots, since it has become evident in the course of the discussion that they play an important part in the disposal of typhoid bacilli.

1. The milky spots and inert particles. — One experiment was made with lampblack, the rabbit being killed one hour after injection. The same features predominated as after injection of bacilli. Under a low magnification the milky spots appear dotted with black (Plate VI., 5), and examination under a high power shows that the pigment is partly contained within cells, while some lies free on the surface. The round macrophages are packed with black granules, and many of the branching cells also contain considerable quantities of pigment.

Comparatively little free pigment can be found over the meshwork, and it appears certain that a milky spot, apart from the phagocytic nature of its cells, is able to fix free particles or bacilli on its surface.

2. The nature of the milky spots. — In its earliest stages a milky spot appears to be formed by macrophages which enter the tissue and become partially fixed as phagocytic branching cells. Small lymphoid cells then appear and group themselves in the center of the spot, followed by the capillaries, which work their way through the meshwork to each milky spot as it increases in size. In short, the fully formed milky spots possess histologically all the elements of a lymph follicular structure, and our studies of the milky spots under pathological conditions have made it evident that they also fulfil the functions of lymph follicular tissue.

It may be mentioned that in the course of these studies we have examined the omentums of about ninety rabbits and seventy-five guinea-pigs.

CONCLUSIONS.

After intraperitoneal injection of typhoid bacilli into a rabbit:

1. The bacilli become fixed in immense numbers on the surface of the omentum.

2. Some may lie free in the fibrinous deposit or over the surface of the milky spots.

3. Some may be contained in macrophages.

4. The bacilli may be rapidly destroyed either extracellularly or intracellularly, or may partly remain intact for some time.

5. If they are rapidly destroyed there is a good microxycyte reaction in four to six hours.

6. If in four to six hours the microxycytes do not arrive in great numbers, secondary centers of multiplication become evident.

7. The secondary centers of multiplication are no longer present in sixteen or twenty-four hours, if the rabbit appears to be recovering.

8. If the rabbit is dying or dies between sixteen and twenty-four hours secondary centers of multiplication are marked features.

9. As with inert particles, the macrophages take the chief part in phagocytosis. Nevertheless, the macrophages cannot effectively dispose of the bacilli unless there is a considerable microxycyte reaction.

10. The stages of degeneration of the bacilli appear to be:—
    1. Pale staining and swollen.
    2. Aggregation of chromatin in center, leaving pale-staining sheath.

3. Small basophile granules.
4. Indefinite eosinophile granules.

1, 2, 3 have been observed to occur extracellularly.

1, 3, 4 have been observed to occur intracellularly.

(We are greatly indebted to Dr. Martha Tracy for the photographs and colored drawings which accompany this series of articles.)

PLATE I.

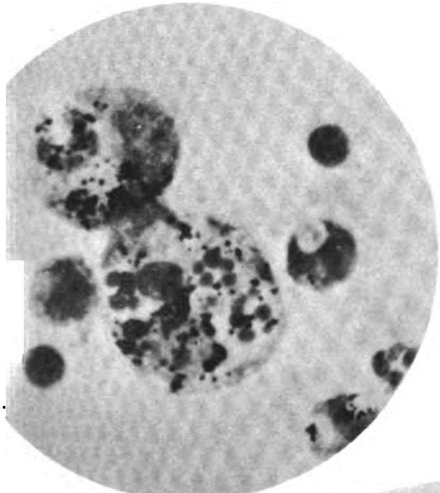
FIG. 1. Lampblack particles in microxycytes. Two hours after injection. x 1,500.

FIG. 2. Macrophages containing microxycytes. Twenty-four hours after injection of lampblack. x 1,000.

FIG. 3. Macrophages containing lampblack particles. Some remains of microxycytes are barely visible. Twenty-four hours after injection. x 1,000.

FIG. 4. Chicken red cells surrounding a microxycyte. The cytoplasm of the chicken cells adjacent to the microxycyte appears eroded. The microscope shows that the central cell around which the chicken cells are grouped is a microxycyte, although it is not clear in the photograph. Four hours after injection. x 1,200.

FIG. 5. Macrophage englobing a number of chicken cells. Twenty-four hours after injection. x 1,200.



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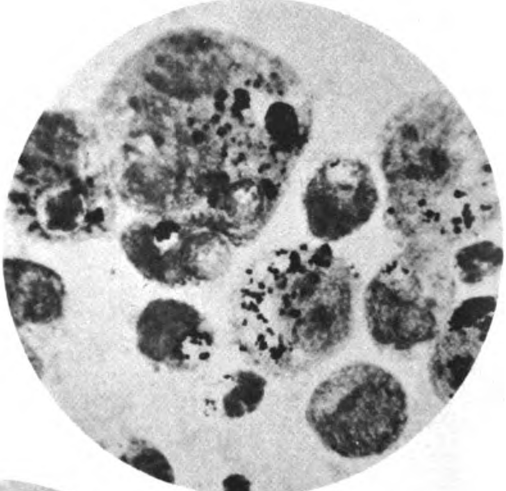

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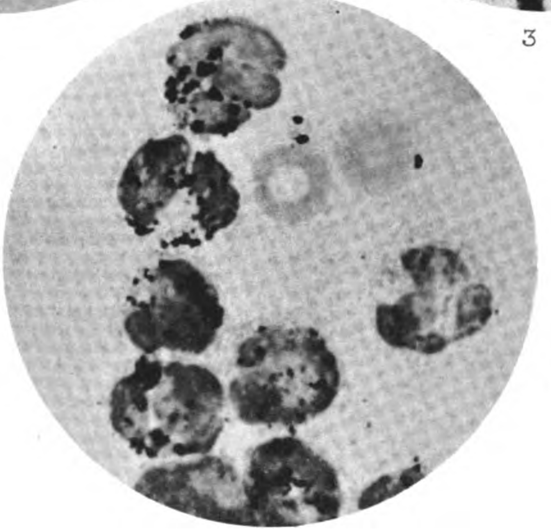

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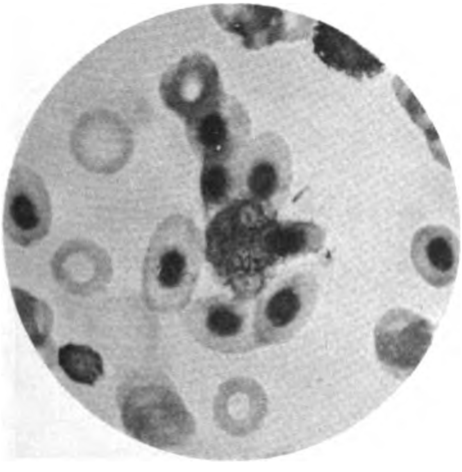

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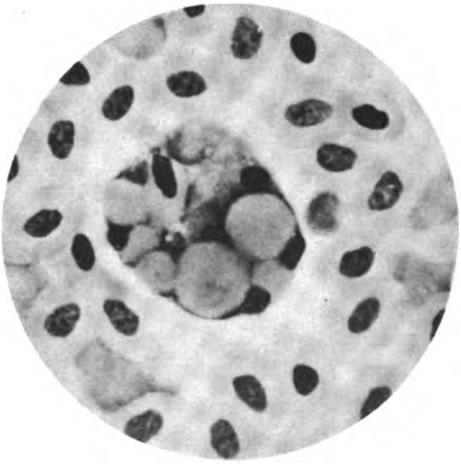

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PLATE II.

(*Sections of anterior mediastinal lymph nodes after injection of lamp-black into guinea-pigs.*)

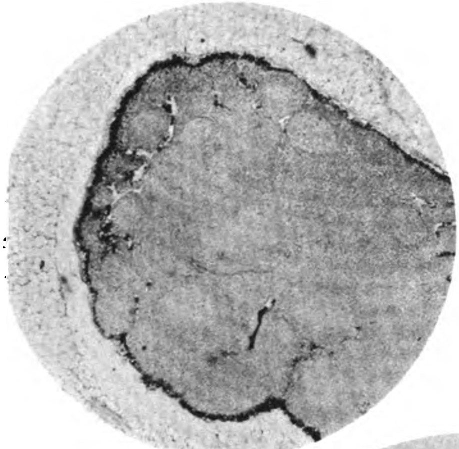
FIG. 1. Fifteen minutes after injection. x 25. The pigment is massed chiefly in the afferent lymphatic plexuses. There is very little in the interior of the node, except in some of the larger sinuses.

FIG. 2. Twenty-four hours after injection. x 25. The pigment lies chiefly in the interior sinuses.

FIG. 3. Fifteen minutes after injection. x 800. From the afferent lymphatic plexus. The pigment lies free in the channels.

FIG. 4. Twenty-four hours after injection. x 800. From an interior sinus. The pigment is chiefly contained in macrophages.

FIG. 5. Fifteen minutes after injection. x 800. Efferent duct of a lymph node filled with free particles.



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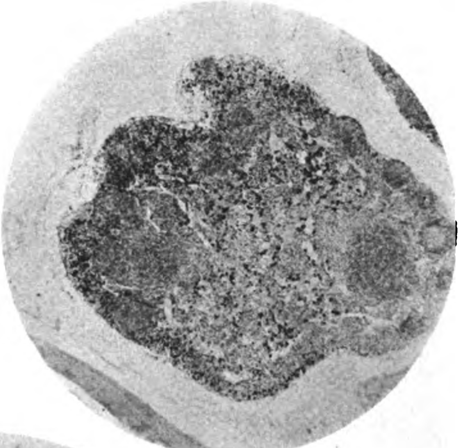

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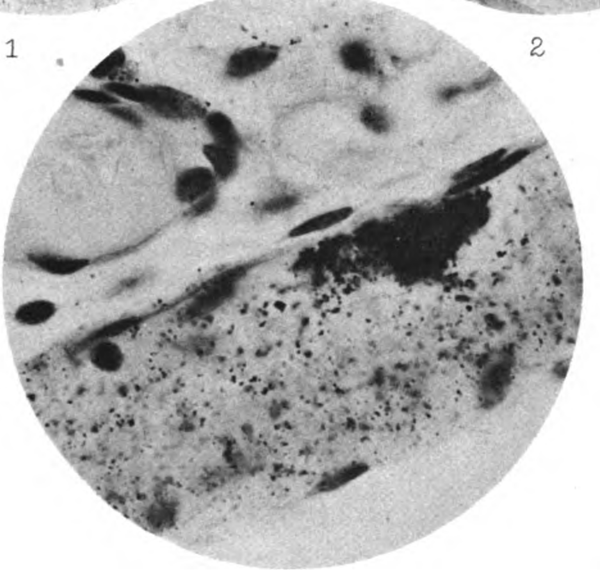

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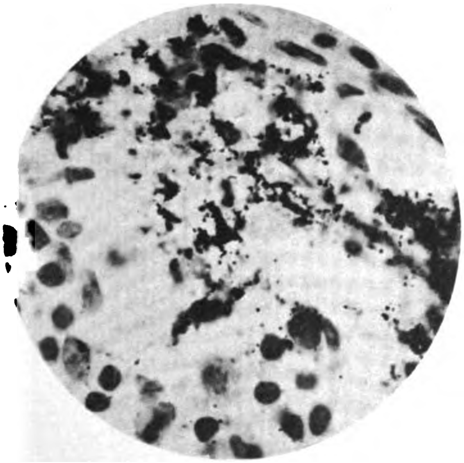

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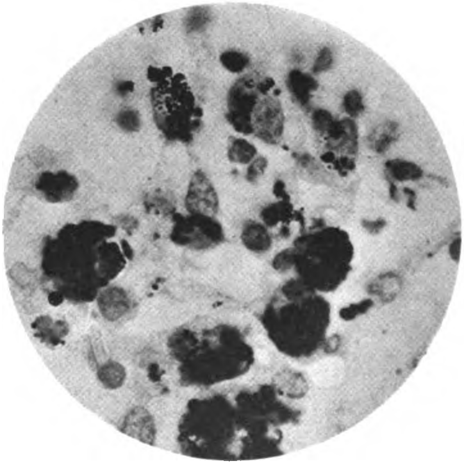

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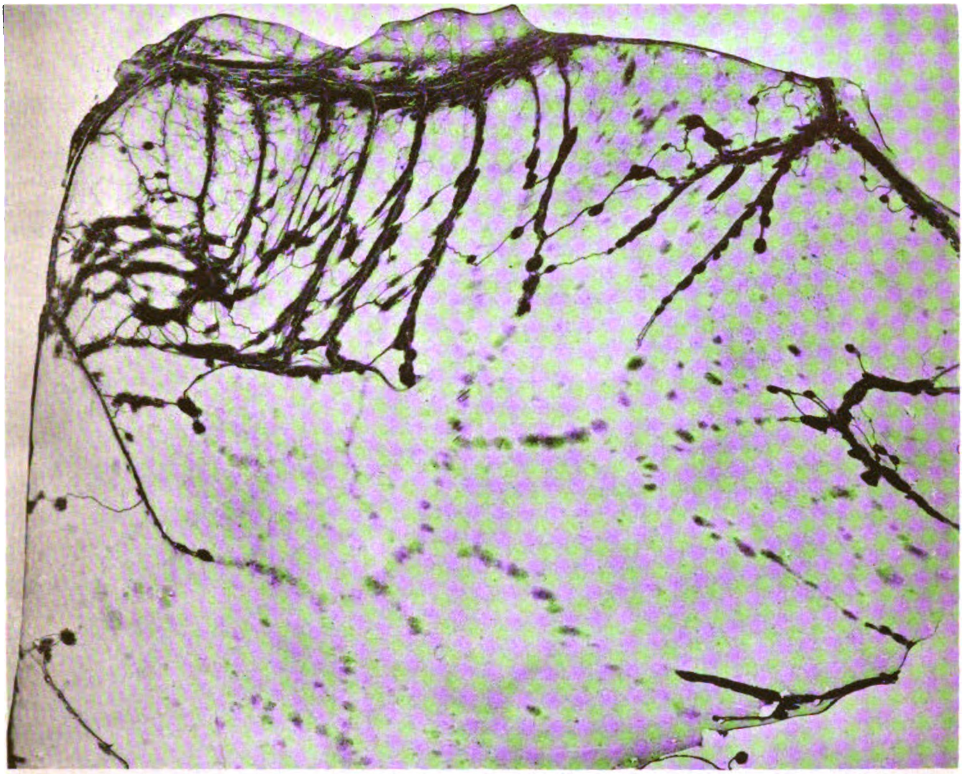
PLATE III.

(*The normal omentum of a young rabbit.*)

FIG. 1. A large omentum spread. x 1½. A tracery work of milky spots indicates the course of the future blood vessels. The capillaries are working their way from the main vessels towards the recently formed milky spots.

FIG. 2. A milky spot without capillaries. x 25. The deeper shade of the center is due to the presence of small lymphoid cells.

FIG. 3. A milky spot with capillary network. x 25. The artery and vein enter the milky spot together and from the opposite side capillaries are leaving the network to work their way towards the next milky spot.



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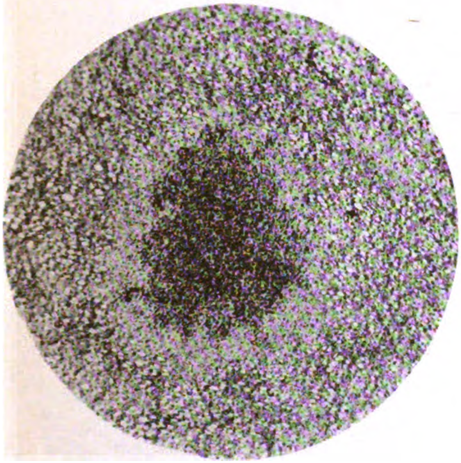

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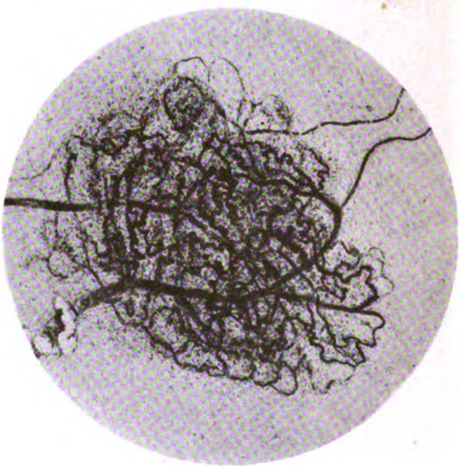

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PLATE IV.

(*The omentum under pathological conditions.*)

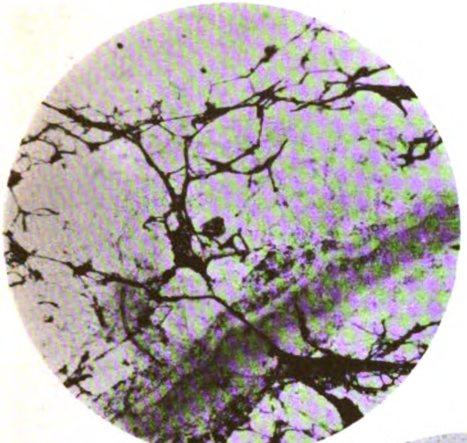
FIG. 1. Lampblack in fibrin network. x 25. Thirty minutes after injection into a guinea-pig. Unstained specimen.

FIG. 2. Fibrinous deposit. Weigert's stain. x 25. Two hours after injection of typhoid bacilli into a rabbit.

FIG. 3. Trailer lying along capillary. x 900. One month after injection of lampblack. The particles have become fragmented and are lying in a vacuole, the outline of which is not distinct. The two long processes of the trailer show clearly.

FIG. 4. Trailer in meshwork. x 900. One month after injection of lampblack. The particles are clearly contained in a vacuole, and are partially fragmented. The nucleus of the trailer is more deeply stained than those of the endothelial cells lying around it.

FIG. 5. Lampblack massed around capillaries. x 50. One month after injection. The particles are all contained within macrophages.



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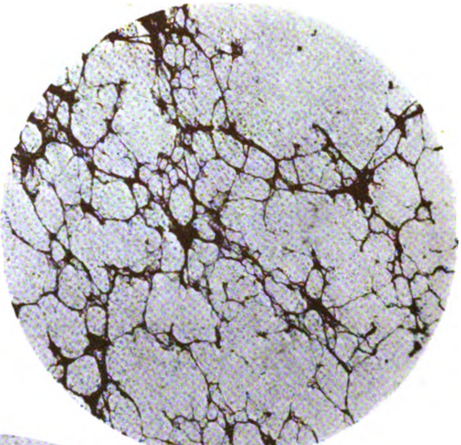

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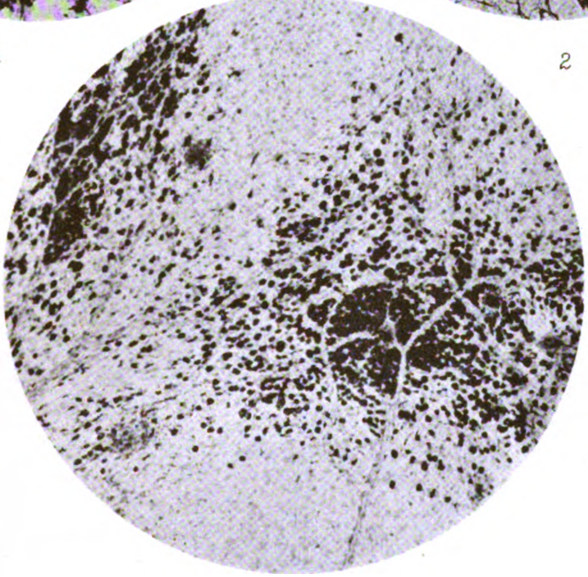

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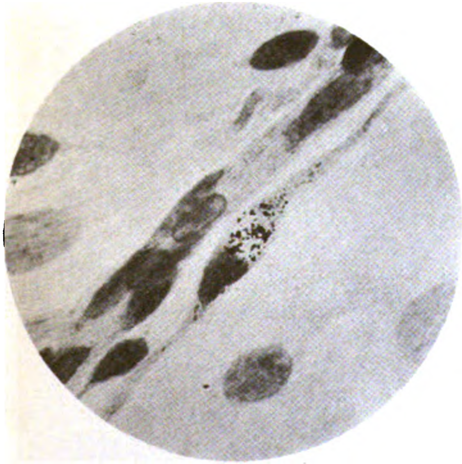

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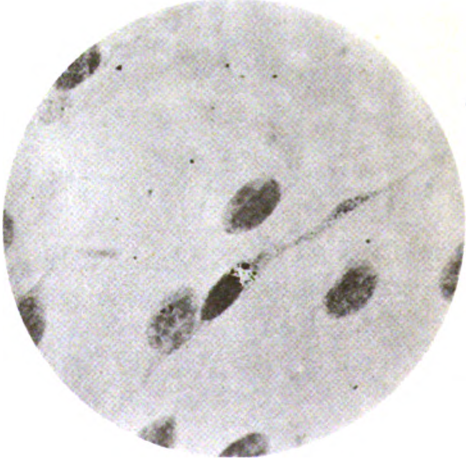

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[illegible]

Fig. 2. [illegible] common [illegible] The [illegible] after [illegible]

Fig. 3. [illegible] centers [illegible] bringing [illegible] the magne-tization [illegible] difference [illegible] original

Fig. 4. [illegible] H [illegible] after an [illegible] H now is a more [illegible] permanent [illegible] also contains [illegible] between these two [illegible] other, the latter is made [illegible] transform itself [illegible]

Fig. 5. H [illegible] must have entirely disapp[illegible]

PLATE V.

(*The omentum under pathological conditions.*)

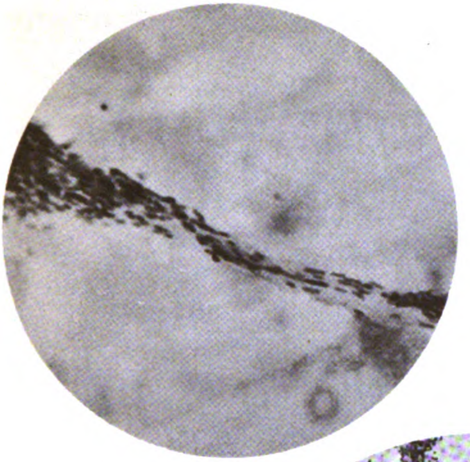
FIG. 1. Typhoid bacilli in fibrin. x 1,000. Thirty minutes after injection into a rabbit. The bacilli lie free on the fibrin, pointing along the long axis of the strand.

FIG. 2. Bacillus coli communis in fibrin. x 1,000. Thirty minutes after injection into a guinea-pig.

FIG. 3. Typhoid bacilli in fibrin. x 1,000. Six hours after injection into a rabbit. Centers of secondary multiplication. Some centers are forming in the interior of macrophages, and some are starting from agglutination clumps, but this difference cannot be made out in the photograph.

FIG. 4. Indigo in phagocytes. x 1,000. Twenty-four hours after injection into a guinea-pig. Below is a macrophage packed with pigment, and above a microxycyte also containing pigment. The trailer between these two cells contains less pigment than the macrophage. Probably the latter is packed so full that it will be unable to enter the tissues and transform itself into a trailer.

FIG. 5. Fibrin attacked by microxycytes. x 50. Twenty-four hours after injection of typhoid bacilli into a rabbit. Both bacilli and granules have entirely disappeared.



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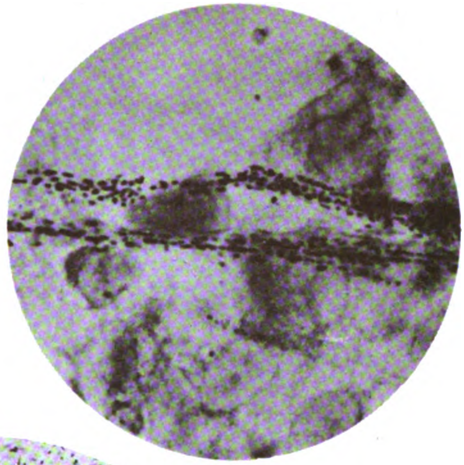

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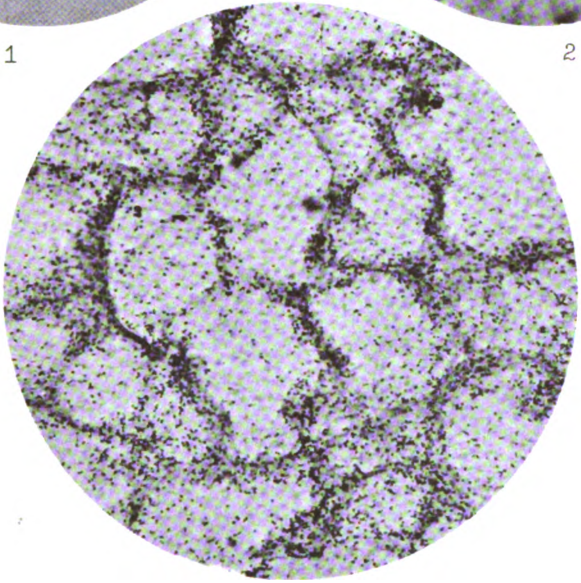

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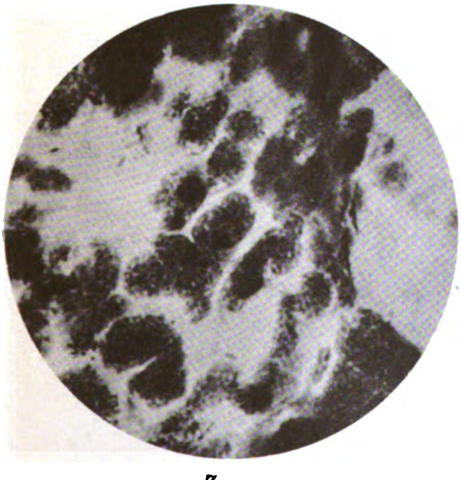

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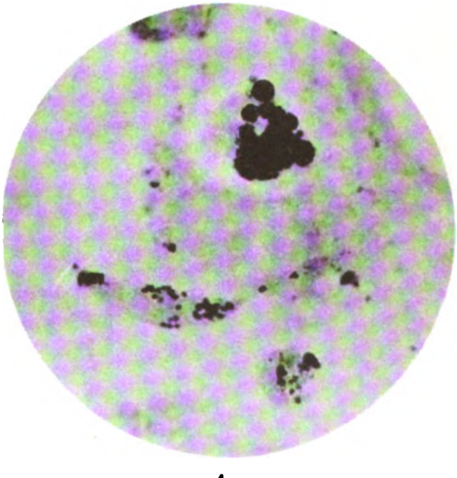

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PLATE VI.

(*The omentum of the rabbit under pathological conditions.*)

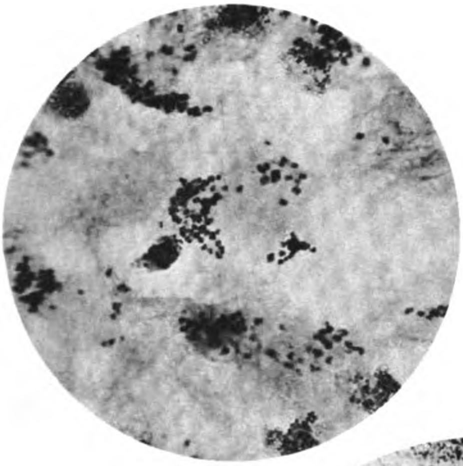
FIG. 1. Large granules in cells of milky spots. x 800. Twenty-four hours after injection of typhoid bacilli. No bacilli or small granules can be seen. The large granules probably represent débris from some previous pathological condition and are not degeneration forms of typhoid bacilli.

FIG. 2. Large granules in trailer. x 800. From the same specimen as Fig. 1. Two branching connective tissue cells without granules can also be seen.

FIG. 3. Typhoid bacilli in macrophages. x 800. From the meshwork of the omentum almost immediately after injection (see Part II., Table IX., 1.). One round macrophage is seen and three early intermediate forms, all containing numerous bacilli. Plate XI., 3, is composite drawing from the same specimen.

FIG. 4. Swollen endothelium. x 800. Sixteen hours after injection of typhoid bacilli. The nuclei are swollen and project into the lumen of the meshes.

FIG. 5. Lampblack over a small milky spot. x 50. One hour after injection into a rabbit. The pigment is massed over the milky spot; very little can be seen on the meshwork.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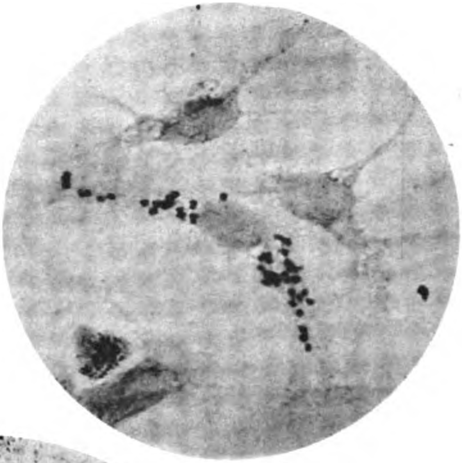
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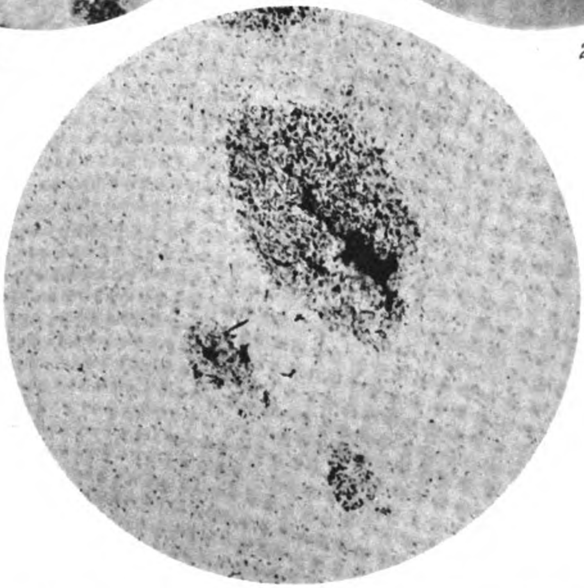
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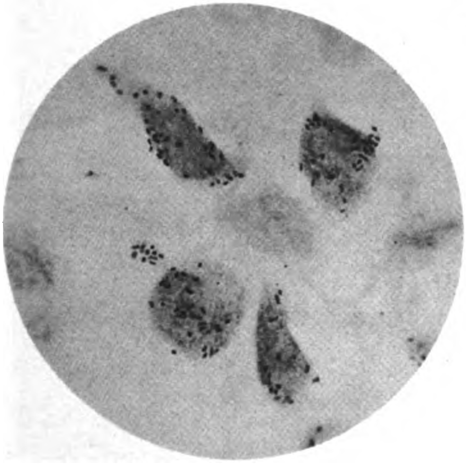
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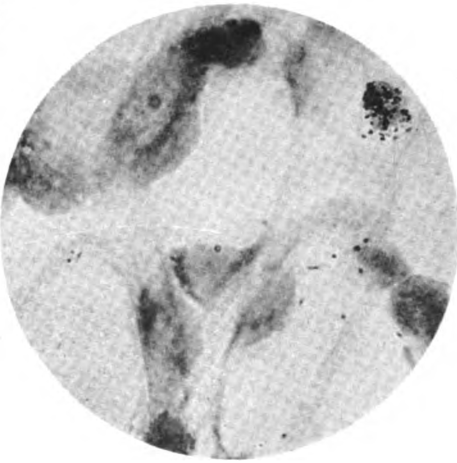
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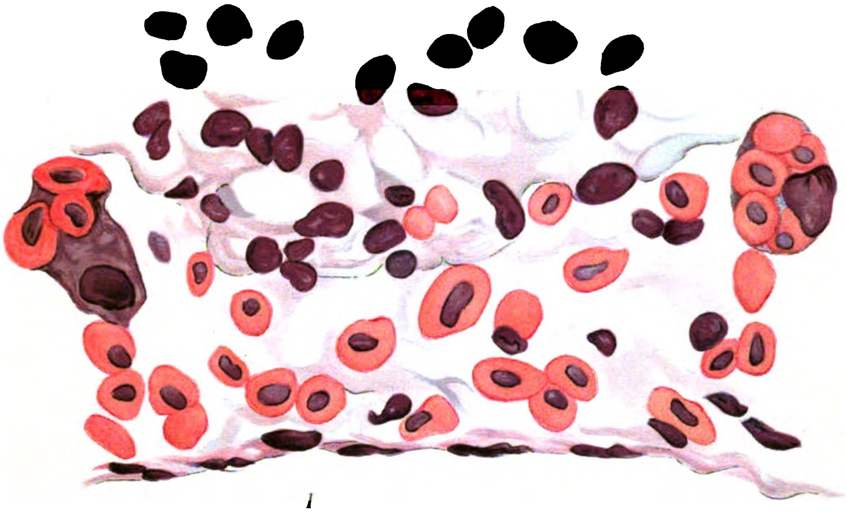
PLATE VII.

(*Sections of anterior mediastinal lymph nodes after injection of chicken red cells into guinea-pigs. Drawn with camera lucida. About 1,400 diameters.*)

FIG. 1. One hour after injection. Afferent sinuses with free chicken red cells. Two macrophages are shown: one on the left just beginning to englobe red cells, and another already stuffed full.

FIG. 2. Eight hours after injection. Chicken red cells englobed by macrophages lying in the interior sinuses. None in the follicular structures. A few free red cells may still be found.



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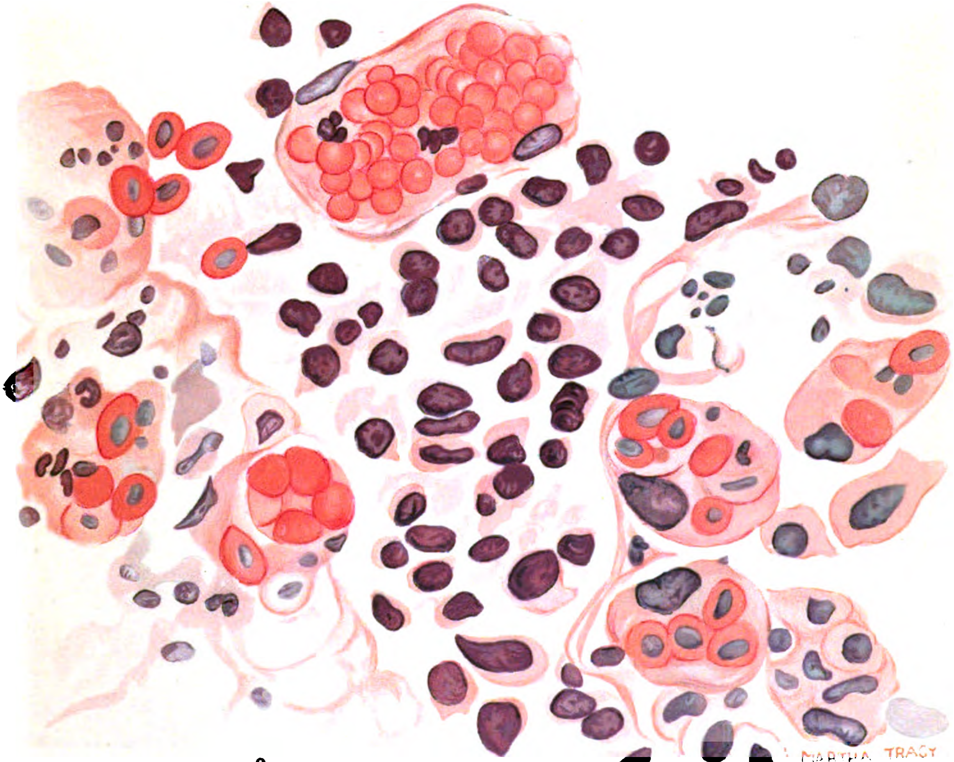

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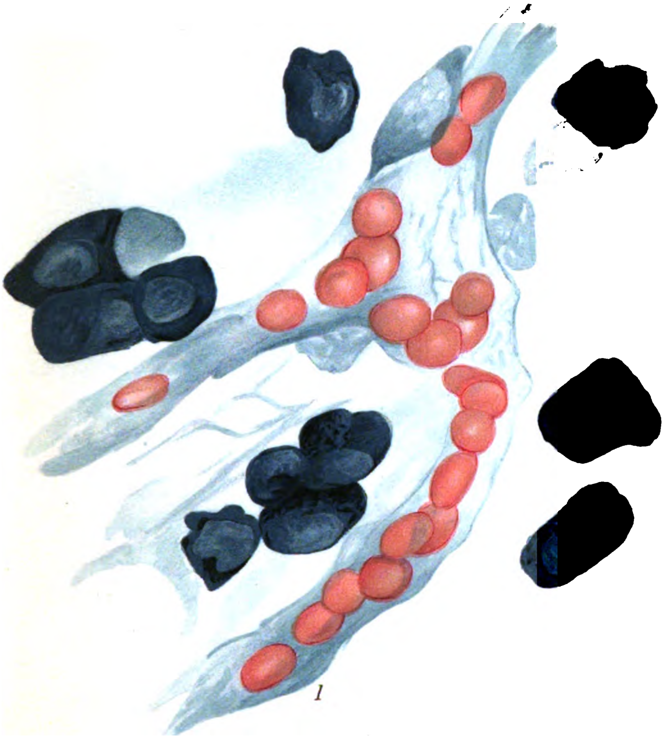
PLATE VIII.

(*Normal structures. Drawn with camera lucida.*)

FIG. 1. From the normal omentum of a rabbit. x 1,400. Basophile lymphoid cells lying around capillaries.

FIG. 2. From the normal omentum of a guinea-pig. A mast cell. x 1,400.

FIG. 3. From a normal milky spot of a rabbit. x 1,000. Macrophages and branching phagocytic cells. One macrophage contains the remains of a microxycyte. A few small red or blue granules can be found in the branching cells.



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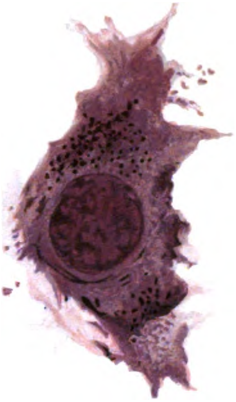

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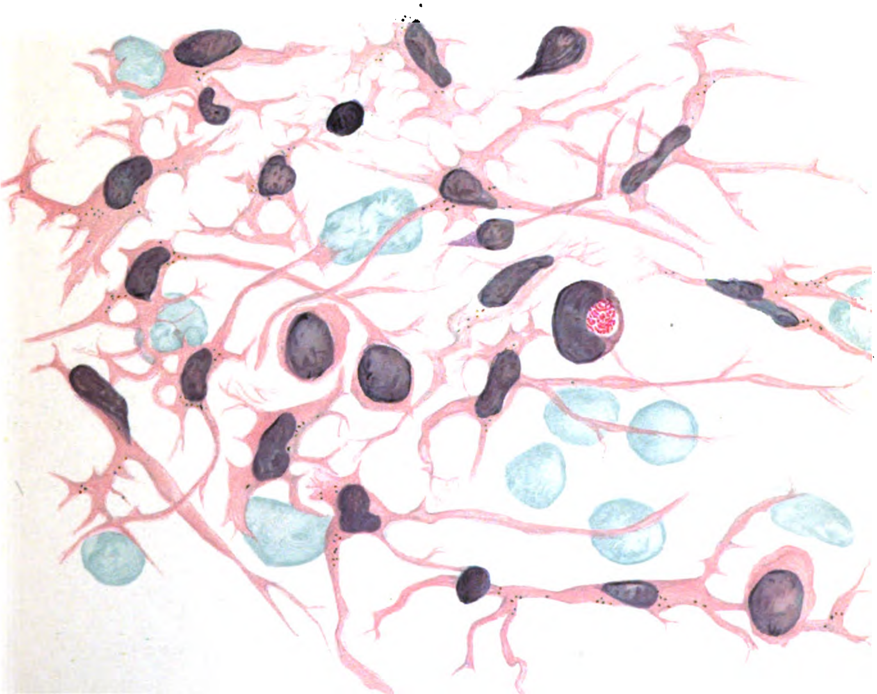

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PLATE IX.

(*Drawn with camera lucida. About 1,400 diameters.*)

FIG. 1. Sixteen hours after injection of typhoid bacilli into a rabbit.

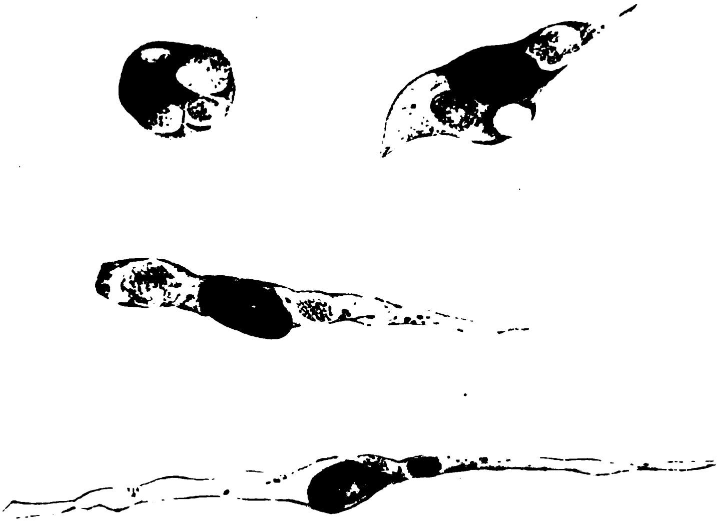
On the omentum, in almost every field of the microscope, phagocytes containing microxycytes could be found. The drawing shows one round macrophage, one typical trailer and two intermediate forms, each containing remains of microxyctes. The cells also contain a few dark granules, representing degenerated bacilli.

FIG. 2. Normal omentum of rabbit showing typical trailer containing small granules, probably derived from an oxyphile cell or extraneous particles.

FIG. 3. From the omentum of a guinea-pig one month after injection of lampblack.

Trailers with vacuoles filled with fragmented particles. In the cell figured on the right the fragmentation is only partial.

Compare with Plate IV., Figs. 3 and 4.



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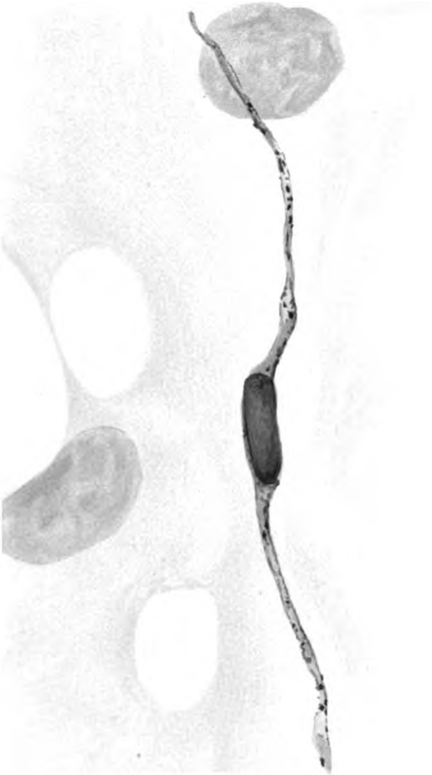

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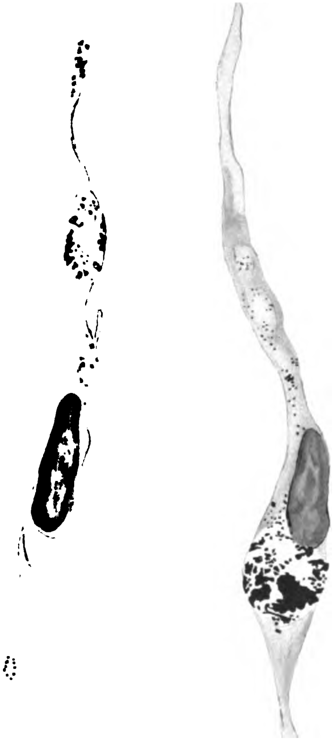

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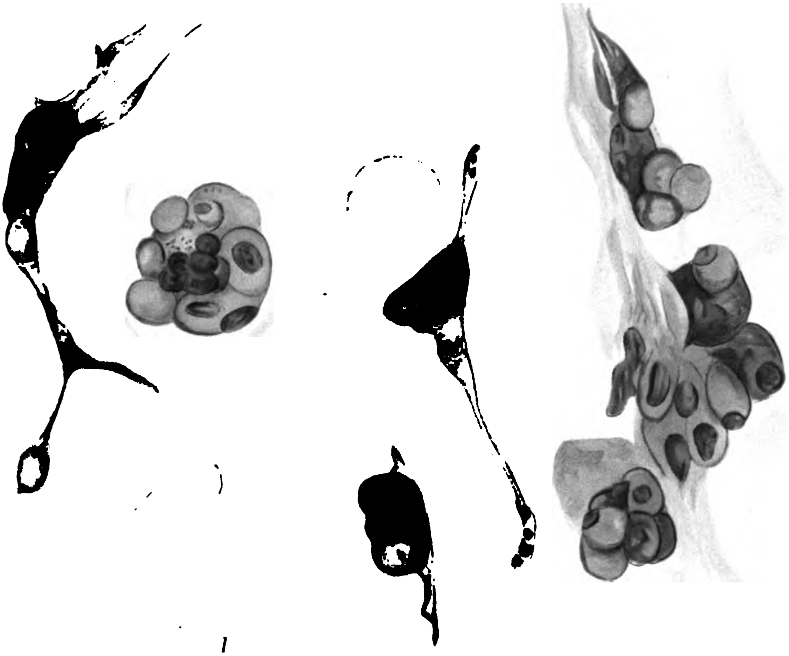
PLATE X.

(*Phagocytosis on the omentum. Drawn with camera lucida. About 1,400 diameters*)

FIG. 1. Five hours after injection of chicken red cells into a guinea-pig. The cells have been englobed by macrophages. On the right is a strand of fibrin, crossing the meshwork, with macrophages clinging to it. In the meshes is figured a large, free macrophage containing chicken cells and remains of microxycytes. Another macrophage is clinging to the wall of the mesh.

In the trabeculæ are two trailers; one containing nuclei of chicken cells, and the other, on the left, with remains of two chicken cells, the nuclei of which are not visible.

FIG. 2. Twenty-four hours after injection of lampblack into a guinea-pig. In the meshwork are figured four phagocytes, showing transitional forms from a round macrophage to an elongated trailer. The phagocytes contain particles of lampblack and remains of microxycytes. Two free microxycytes and one eosinophile cell, all containing a little pigment, are also figured.



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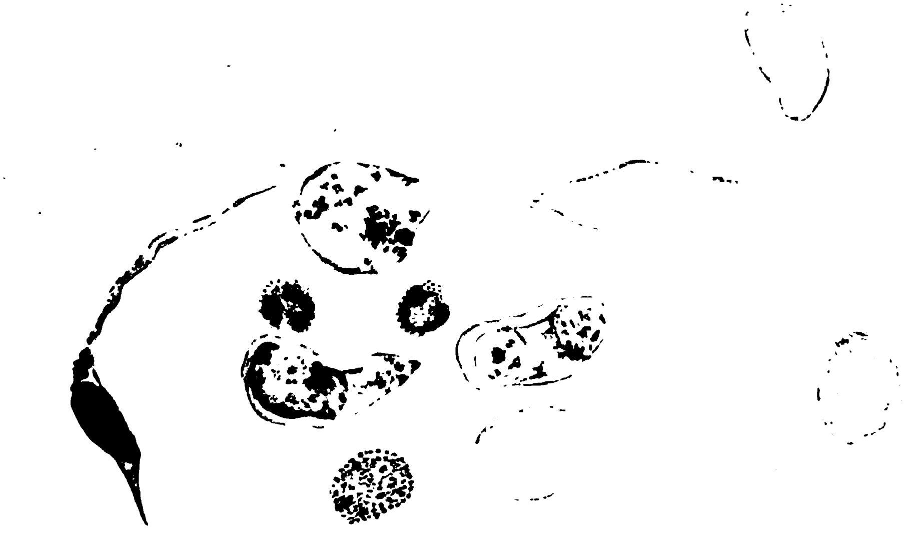

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PLATE XI.

(*The omentum immediately after injection of typhoid bacilli into a rabbit. Three types of what may occur. Drawn with camera lucida. About 1,400 diameters.*)

FIG. 1. Explosive extracellular destruction of bacilli.

The bacilli lie free in the meshwork. They have undergone a peculiar form of degeneration, the chromatin being massed in the center of each bacillus, leaving a faintly staining sheath.

See Part II., Table IX., 7. *Wash*, 1,000. *Organs*, 0.

FIG. 2. Explosive intracellular destruction of bacilli.

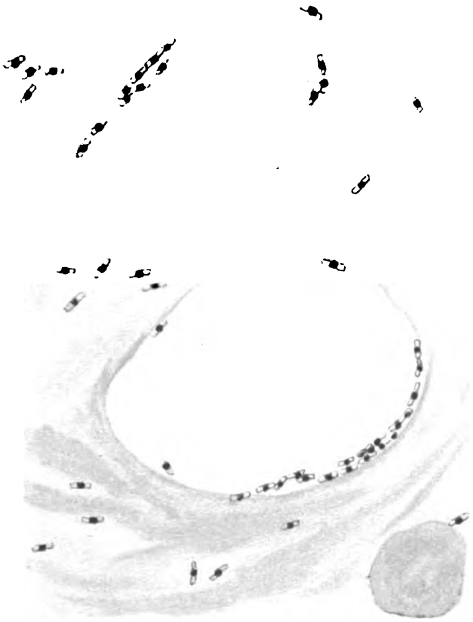
From the edge of a milky spot the branching cells are filled with indefinite reddish and blue granules representing the remains of bacilli. No free bacilli or granules.

See Part II., Table IX , 5. *Wash*, 350,000. *Organs*, 0.

FIG. 3. No explosive destruction of bacilli. Composite drawing. Meshwork showing a macrophage, intermediate forms, and a trailer, all containing intact bacilli.

See Part II., Table IX., 1. *Wash*, many millions. *Organs*, full of bacilli. Compare with Plate VI., 3, from same specimen.

Note. — In each figure a few pale endothelial nuclei may be seen.



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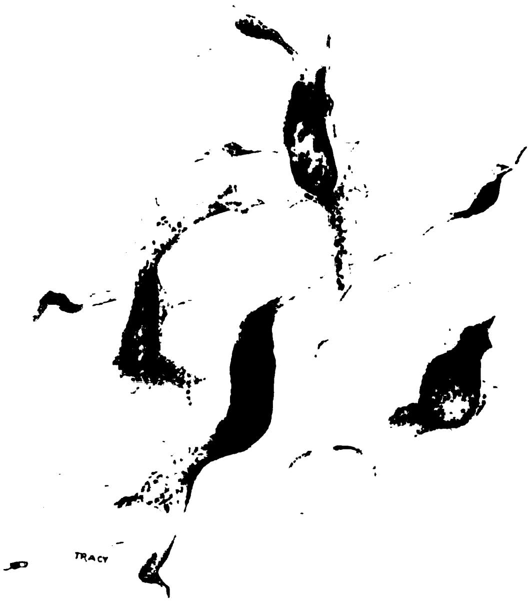

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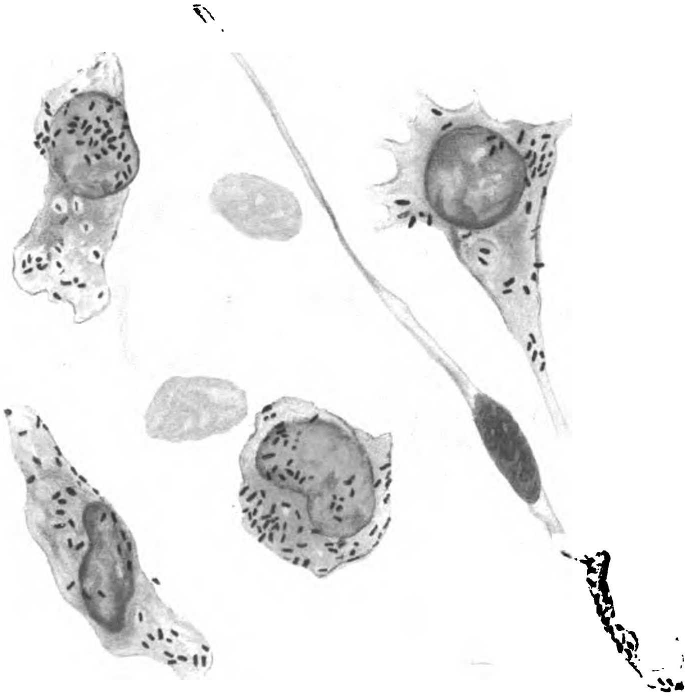

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MICROSCOPIC EVIDENCES OF ABSORPTION IN THE LARGE INTESTINE.*

JOHN LEWIS BREMER.

(*From the Histological Laboratory of the Harvard Medical School.*)

In 1900, Pio Mingazzini,[1] in a series of articles, established the fact that food is absorbed in the small intestine by a process which can be demonstrated microscopically. Before this several observers had noticed that the epithelial cells covering the intestinal villi were often, in sections, seen to stand away from the underlying tissue, leaving spaces between the two, sometimes apparently empty, more often filled with coagulated material. These spaces were not observed in other epithelium, nor always in the villi of the small intestine. In 1887 Grünhagen[2] published a paper stating that at the end of each villus there was a pore, and that this coagulated mass was the food taken in through this pore, ready for absorption by the veins or lacteals, and meanwhile present in such quantity as to dissect away the epithelium to make room for itself. The following year Heidenhain[3] made the sweeping statement that all the appearances noticed were due to the shrinkage of the connective tissue core of the villus, and were to be attributed to the fixing fluids. The space caused by this shrinkage was naturally filled by the body fluids, which might remain clear, or form a granular precipitate or become reticular. All following authors agreed with Heidenhain, and some made the further suggestion that the reticular appearance so often seen, like a number of small rings close together, was to be accounted for by the fact that the section had included a portion of the wall of the villus away from the observer, who was thus looking on the bases of the cylindrical epithelial cells.

Mingazzini showed conclusively that these appearances were part of a cycle of food absorption. The epithelium in the normal resting condition consists of a single row of

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cylindrical cells, resting on a basement membrane, which in turn is attached to the underlying reticular or connective tissue core of the villus. Each cell has its nucleus in the lower half of the cell, and is provided with a top plate; the protoplasm is homogeneous throughout the cell. Here and there are mucous or goblet cells. The first change after the introduction of food into the intestine is a differentiation in the protoplasm, that below the nucleus becomes more granular, more irregular. Then the nucleus moves toward the top of the cell, and may sometimes undergo specific changes, such as becoming rounder or more rod-shaped. Then there are seen clear spaces, vacuoles to all appearances in the stained specimen, in the sub-nuclear portion of the cell; these clear spaces become larger, causing the sub-nuclear end of the cell, and thus the whole cell, to become longer, until this lower end of the cell is composed of nearly clear globules, outlined by the protoplasm. At a later stage the end of the cell breaks off, and these globules or spherules, each surrounded by a thin wall of protoplasm, are set free in a space between the basement membrane and the shortened body of the epithelial cell. The appearance then is as though the epithelial layer had been dissected away from the basement membrane. Soon these spherules break down into a granular material, which finds its way, not to the lacteals, but to the veins situated close to the basement membrane. The shortened epithelial cells, meanwhile, have reconstructed their broken, often ragged proximal ends, renewed apparently their lost protoplasm and, as the granular material is removed from the intervening space, settle down again upon the basement membrane. The return of the nucleus to its former condition and position makes them again normal resting cells. In addition to this method, by which food material is passed from the intestine into the veins, a sort of reversed secretion, the part played by leucocytes is emphasized by Mingazzini; these are very numerous in these tissues, take up some of the food material, and return with it to the veins.

Macallum[4] in an article on the absorption of iron, though writing previous to Mingazzini, curiously confirms his work, for he finds that the iron solutions, particularly the "proteid irons," are situated after ingestion in the sub-nuclear portions of the intestinal epithelium, and in the leucocytes, whether these are between the epithelial cells, or in the connective tissue below; and that later the solutions are found in the veins, not in the lacteals; they are present in the red and the white corpuscles, and in the plasma. Macallum's figures, while intended merely to show the distribution of iron, are quite in accord in some cases with those of Mingazzini, for we find the enlargement and vacuolization of the sub-nuclear portions of the cells, and the irregular bases of cells which are not in contact with the basement membrane. Macallum points out that the appearances are not the same in the case of iron held by fats, as when the "proteid iron" is used, and Drago,[5] working in the same laboratory with Mingazzini, Reuter,[6] and others, also show that fats are absorbed in a perfectly distinct manner.

I have given this brief summary of the previous work in this line because the present paper is based directly upon it, and pretends merely to enlarge slightly upon the facts already known. The villi of the small intestine, especially near the tips, have, in all previous cases, been chosen for the investigation of this absorption, except that Macallum, while searching for the iron in other tissues, as in the liver and kidney, found in the bases of Brunner's glands of the duodenum the iron in the sub-nuclear portion of the epithelium. This he passes over with a statement that the iron is evidently about to be excreted by the gland. This year my attention was called to similar appearances in the large intestine of man. On examination of a number of specimens of large intestine, animal as well as human, and comparison of these with the plates of Mingazzini and with actual specimens of small intestine, I find in by far the greater number of cases some stage of spherule formation. One difference between the process as seen in the large intestine and that seen in the small intestine is that in the large intestine the cells in activity lose the top-plate, which is never so marked as in the cells of the small intestine, and that with the return to the resting stage this top-plate also reappears. The large intestine is not provided with villi, but the process takes place over the surface epithelium and also in the glands, even down to their fundus. It may be objected that the food material could not find its way into a gland from which mucin was being poured, and in fact it was found that the activity of absorption was greater the fewer mucous cells there were. As in the case of the villi of the small intestine, these appearances have naturally been noticed before in the large intestine, but have always been attributed to the shrinkage of surrounding parts.

With the view of gaining more definite knowledge in regard to this absorption, I have examined the intestinal tracts of several animals, monkey, cat, rabbit, guinea-pig, and others. In certain cases the animals were fed on known material and killed at known intervals after feeding; for the knowledge in regard to the time after feeding when the food should be found in the various parts of the intestinal tract I am indebted to Prof. W. B. Cannon.[7] The specimens were said to show spherule formation (1) when the spherules were present free underneath the cells; (2) when the spherules were in the subnuclear portion of the elongated cells; or (3) when the cells showed combined the absence of top-plate, the displacement of the nucleus toward the free surface, and a distinct differentiation of protoplasm, with the sub-nuclear zone more reticular and granular. Care was taken that only the epithelium cut in good cross section should be studied; and places where there were many leucocytes among the epithelial cells were considered doubtful, for the protoplasm of the former often stains a lighter tint, and so might be mistaken for a spherule when the nucleus was not in the plane of section. That these spherules are not mucin in process of formation was shown by differential stains. The results may be stated as follows: In the fasting animal, with no food in the large intestine, all of the cells are in the resting condition; in one fasting cat (twenty-four hours without food), in which food was present in the large intestine, the process of spherulation was going on to a small extent. In all the other cases, when food containing proteid was present in the large intestine, the process was found. Moreover, in the rabbit and the guinea-pig, whose large ceca are almost always full of food, the same process was seen in the cecum. From finding evidence of absorption in the rest of the intestinal tract below the stomach, even at the fundus of the glands, I have no doubt that Măcallum's figure showing iron in the bases of cells in Brunner's glands of the duodenum really means that absorption, not excretion, of iron is going on there.

The process in the large intestine and cecum, though comparable in other ways with that in the villi of the small intestine, is far less active. We find no long patches of epithelium, every cell of which contains spherules, nor any such great accumulation of the spherules below the epithelium. Many glands show perfectly normal resting cells, or are lined almost entirely by goblet cells, pouring out mucin. Then in perhaps a dozen cells, sometimes near the fundus of the gland, sometimes in the surface epithelium, the spherule formation will be found. This must mean that the process is going on to a smaller extent and more slowly than in the small intestine. We should expect that it would be a slower process, for the food stays in the large intestine (and in the cecum in those animals provided with large ceca) much longer than in the small intestine, and it seems probable from my specimens that absorption goes on as long as food remains in contact with the wall. That the process goes on to a smaller extent is confirmed by the work of Harley.[8] He removed the large intestine and sutured the small intestine to the rectum, and found that there was no decrease in the absorption of fats or of carbohydrates, but that the animal absorbed one-tenth less proteid material. This one-tenth of the proteid was presumably absorbed by the large intestine, the remaining nine-tenths by the small intestine, so that we should expect the activity shown by the cells of the large intestine to be less.

Harley's work and that of Macallum with the proteid and iron make it almost certain that the food entering the body by this method of spherule formation is proteid; fats can be observed passing through the cells in another form, perfectly distinct from that we are considering, and carbohydrates are not absorbed in the large intestine, where the spherules are found. As Cannon[9] has shown that enemata do not normally pass beyond the ileo-cecal valve, unless given in large amounts, we have here a histological confirmation of the clinical fact that nutrient enemata should consist of proteid material, and that any drugs contained should be combined with proteids.

As a control on my ideas of normal resting cells, I examined fetal small and large intestines. No appearances like those found in the active cells were noticed. Then I examined the intestinal epithelium of two sets of opossums, one of 10.5 millimeters taken from the uterus, the other of 12.5 millimeters taken from the pouch, already attached by the mouth to the nipple. In the embryos the cells were normal and resting, while in those that had already taken milk into the intestine the bases of the cells showed in several cases irregularities of the protoplasm, and in some instances distinct spherules.

CONCLUSION.

Food is absorbed in the large intestine (and in the cecum of those animals with large ceca) in the same way as in the small intestine, but to a smaller extent. The process goes on in the epithelium of the surface and in that of the glands, but seems to be retarded or stopped if the mucous cells become very active. The same cycle of spherule formation is seen in the epithelial cells; absorption by leucocytes is also present. This seems to represent the absorption of proteids.

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DESCRIPTION OF PLATES.

PLATES XII AND XIII.

FIG. 1. (From Mingazzini.) Villus of the small intestine of a hen, in state of repose. The protoplasm of the cylindrical cells is homogeneous, and the cells rest on the basement membrane. oc. = top-plate; sv. = connective tissue stroma of the villus; mv. = muscle of the villus; mb. = basement membrane; lc. = lymph channel.

FIG. 2. (From Mingazzini.) Villus of the small intestine of a hen, in functional state. The apical portion contains the secretion. sa. = internal secretion; cs. = blood capillary; cl. = leucocytes among the cylindrical epithelial cells. The other lettering same as above.

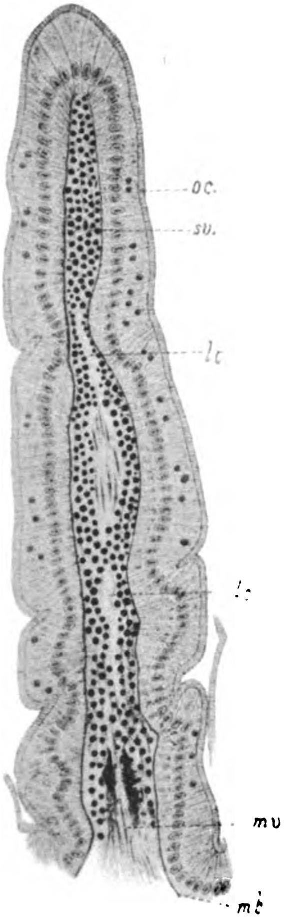
FIG. 3. (From Mingazzini.) Villus of the small intestine of a hen in almost complete functional activity. In the apical portion absorption has already taken place and the cells are again in contact with the basement membrane; near the apex the maximum activity is seen, with a gradual diminution toward the base of the villus. zs. = dark zone of the absorbing cells; zc. = light zone. The other lettering same as above.

FIG. 5. (From Mingazzini.) Portion of the epithelium of a villus in activity, showing the bases of the cells transformed into spherules.

FIG. 6. (From Mingazzini.) Portion of the epithelium of a villus in activity, showing the spherules already separate from the cells, and the ragged bases of the latter.

FIG. 7. Large intestine of guinea-pig, killed three hours after feeding. A part of the surface epithelium and the neck of a gland are shown. Int. = lumen of intestine; Lu. = lumen of gland; G.c. = goblet cell; B.m. = basement membrane; R.t. = reticular tissue; L. = leucocyte; Sph. = spherules of food material; N. = rod-shaped nucleus.

FIG. 8. Human large intestine, in activity. The epithelium of one side of a gland is shown, with the reticular tissue stroma on which it lies when in a resting state. Ep. = epithelium; R.t. = reticular tissue; Lu. = lumen of gland; G.c. = goblet cell; T.p. = top-plate, regenerating in lower part of gland; L. = leucocyte; Sph. = spherules of food material, now broken off from cells, leaving ragged bases; Gr. = granular material formed by the breaking down of the spherules; Ve. = capillary veins, containing the granular material.



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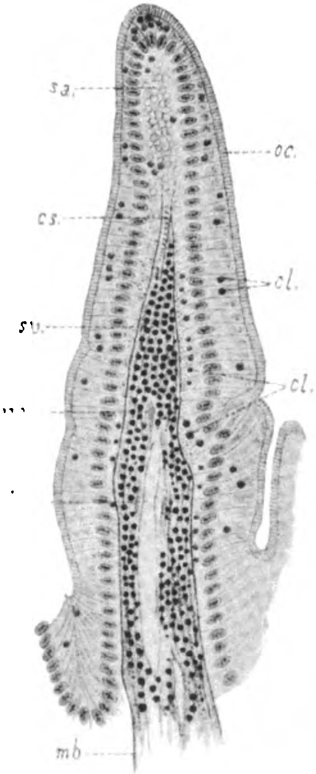

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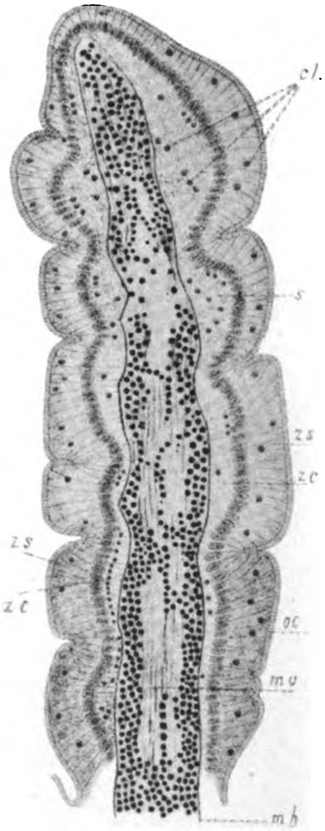

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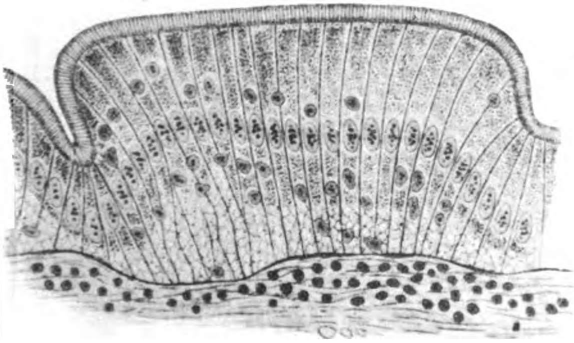

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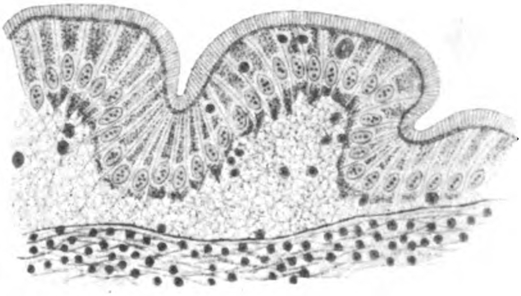

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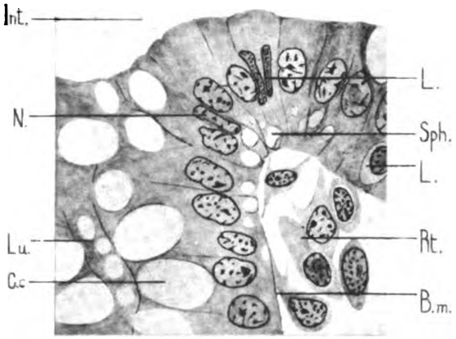

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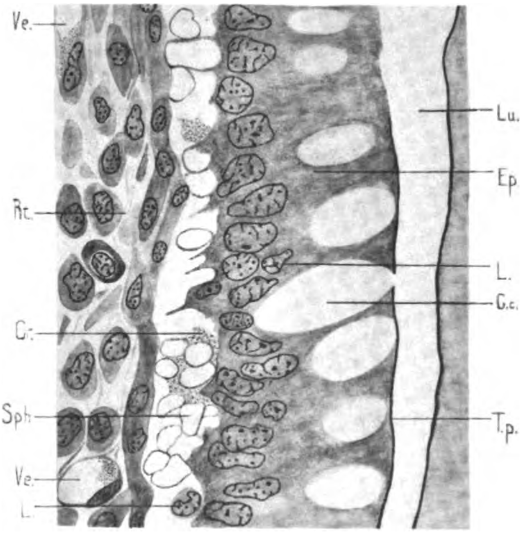

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TWO MODIFICATIONS OF THE LEISCHMAN STAIN.*

Ross C. Whitman, A.B., M.D.

For the last year or more I have been experiencing considerable difficulty from the deterioration of my Leischman stains. Wright's stain is the form in which I have used it chiefly. A specimen of this stain, which when freshly prepared gives an absolutely ideal picture, has often in the course of a few days altered to such an extent that it could no longer be used. Since the publication of Hasting's stain, I have ascribed this fact to impurities in the methyl alcohol used (Merck's highest purity), and this has led me to try whether the same result could be accomplished without the use of methyl alcohol. I have succeeded in doing this in two ways, which will, I think, be of some general interest.

For the first I need only say that I have substituted as a solvent for Wright's stain the mixture of glycerine and absolute (ethyl) alcohol first proposed, if I remember correctly, by Professor Simon for the Jenner stain. I have used a mixture of one part glycerine and nine parts alcohol. The mixture is not an ideal fixing fluid, but if one uses methyl alcohol as a fixing fluid and follows this with the stain diluted at once with water, no time is lost in the staining. The solution so prepared is quite permanent. Ordinary wood alcohol may be used as a fixing fluid, thus obviating the expense of the pure spirit. Since the stain is more soluble in the glycerine alcohol mixture than it is in the methyl spirit, a relatively larger amount of water should be used to dilute it than in the case of the methyl alcohol solution. About five drops of the stain to eight drops of water I have found to be satisfactory. The staining is more rapid and intense with this solution than with the regular Wright's stain.

The second modification may be used as an emergency stain or as a test in the manufacture of a Leischman stain

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to determine whether the methylene blue contains the proper amount of methylene azure. The blood smears may be fixed in any convenient way, except that I have found formalin alcohol unsatisfactory, probably on account of its acid reaction. A saturated watery solution of bichloride of mercury gives very satisfactory results and fixes in two or three minutes. For the staining any polychrome blue may be used. I have employed indifferently a polychrome blue made according to Wright's formula, and old Lœffler's alkaline blue, and an Unna's polychrome blue. The staining procedure with the Lœffler blue is as follows:

1. Immerse the smear for one minute in the Lœffler blue.
2. Without washing transfer to a solution of water-soluble eosin about 1–2000, for fifteen seconds.
3. Without washing, again transfer to the methylene blue, for fifteen seconds.
4. Wash and clear in distilled water.

The staining time will vary somewhat with the strength of the two solutions, success with the stain being merely a question of getting the proper balance of the two stains. The nuclei should be stained a bright cherry red, the protoplasm of mononuclear cells a pale blue, and the granules should stand out very clearly and distinctly. The color of the red cells depends upon the amount of methylene azure in the blue and the amount of clearing. Unna's polychrome blue may be used alone without any eosin at all and gives the red cells a bright red color. Specimens so stained, however, have the untidy appearance of an over stained specimen and the stain is objectionable on this account.

In this brief note precise references to the literature have been purposely omitted as unnecessary. I trust, however, that the fact above noted may be found useful to others beside myself in blood work.

REGENERATIVE CHANGES IN THE LIVER: A STUDY OF EXPERIMENTAL LESIONS IN THE DOG.*

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(Conducted under a grant from the Rockefeller Institute for Medical Research.)

Elsewhere[1] I have described a form of experimental cirrhosis which in its nature is essentially a repair of extensive but more or less irregularly arranged necroses produced by the action of hemagglutinative sera. To this account the present communication is more or less supplemental in that it deals with some of the more important of the finer details of the regenerative process, which, for the sake of brevity, were merely outlined in the earlier communication.

The reparative process to be described is of peculiar interest in that it follows definite necrotic lesions produced by an agent reaching the liver through the circulation and not those caused by direct mechanical injury. For this reason, and also because of the resemblance of the early necrotic lesions to acute yellow atrophy and of the later reparative process to cirrhosis, a comparison with the regenerative processes in the liver of man is possible.

Our knowledge of the regenerative changes in the liver is based mainly on the studies of the repair following mechanical injuries and that occurring in acute yellow atrophy and, to a lesser extent, in cirrhosis of the liver. The principal experimental studies[2] since the introduction of aseptic methods and the development of our knowledge of indirect cell division are those of Podwyssozki,[3] Ponfick,[4] and v. Meister.[5] A comprehensive review of the reparative process in acute yellow atrophy may be obtained from the writings of Meder,[6] Marchand,[7] Stroebe,[8] Barbacci,[9] and MacCallum,[10] while the regenerative changes in cirrhosis, first emphasized by Kretz,[11] have since been studied by MacCallum[12] and Kelly.[13]

Podwyssozki, who has made the most comprehensive study

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of the repair of wounds of the liver produced in various ways, concludes that small losses of substance are repaired, without the formation of fibrous tissue, by proliferation of the liver cells and the cells of the small bile ducts. In more serious injuries in addition to such proliferation a more or less abundant formation of connective tissue occurs. In his studies he used the rat, cat, rabbit, and guinea-pig. The formation of new liver cells by mitosis begins after twenty to forty-eight hours and mitotic figures are very abundant by the fourth to the seventh day. Mitoses are also seen in endothelial and connective tissue cells and in Kupffer's cells. The newly formed liver cells are usually large and pale and of round or oval shape. The bile ducts, especially the interlobular, proliferate freely and from them new liver cells may develop directly or they may form large, irregular sprouts in which multinucleated masses of cells appear and from which new liver cells separate. Podwyssozki emphasizes the similarity of the repair of liver cells in pathological conditions to their embryonic development.

Ponfick's experiments, which were repeated by v. Meister, demonstrate that after extirpation of large portions, three-quarters to four-fifths, of the liver of the rabbit a hyperplasia of the remaining substance causes a restoration of the liver to its original size. This proliferation, which is characterized by the formation of lobules of unusual size, is a condition analogous apparently to the vicarious hypertrophy of paired or multiple organs and is due almost entirely to a regeneration of liver cells. Mitoses were first observed after forty-five to seventy-two hours and soon became very abundant. Large, pale, finely-granular cells, often with two nuclei, were very numerous and apparently represent newly formed cells. In the bile ducts and blood vessels also was seen evidence of proliferation, though mitotic figures could not be readily demonstrated.

The so-called adenomatous nodules ("multiple knotige hyperplasie") described by many investigators, and studied especially by Marchand and Barbacci in connection with acute yellow atrophy and cirrhosis, may be considered as the result of a hyperplasia comparable to that produced experimentally by Ponfick and v. Meister. A similar hypertrophy of massive character has been observed by Ponfick [14] in association with echinococcus cyst.

Practically all who have studied the repair process in acute yellow atrophy are in accord concerning regeneration by mitosis of surviving liver cells, but some difference of opinion exists in regard to the solid and tubular cell-strands usually described as newly-formed or pseudo bile ducts. All recent investigators have abandoned the idea that these structures are atrophied rows of liver cells and consider them as sprouts from the small interlobular bile ducts growing into the necrotic lobule. By a gradual transformation of the cells of such sprouts true functioning liver cells are formed. Marchand, however, states that similar structures may also be formed by the proliferation and fusion of the true liver cells in small islands of liver substance which have escaped the general destruction. This latter view Stroebe considers unproven and MacCallum in the one case which he studied was not able to demonstrate this form of proliferation.

The recent studies of regenerative changes in cirrhosis of the liver were undoubtedly suggested by Kretz's statement that this disease is the result of successive processes of repair following repeated focal degenerations of the parenchyma. These studies treat of changes in the size, shape, and internal arrangement of the lobule, of its altered blood supply, and of the origin and appearance of the newly-formed cells. In general, the proliferation is similar to that in acute yellow atrophy, but, owing to the chronicity of the lesion as seen at autopsy, mitotic figures are rarely found in the parenchymatous cells. The new formation of such cells, however, is indicated by the occurrence among cells of the usual appearance of large, clear, round, plump cells with two or more nuclei and devoid of fat and pigment. What has been said of the newly-formed bile ducts in acute yellow atrophy holds for the similar structures found in cirrhosis.

The present study is based on material from the livers of sixteen dogs representing periods varying from thirty-eight hours to thirty-six days after the injection of the serum. As the larger portion of the animals died during the first week, it is possible to present an accurate account of the sequence of reparative changes to the end of the seventh day. The late changes are also well represented by those animals which survived the acute disturbance. The middle period, however, is not well represented.

The degree of repair has been found to vary considerably at any given period. This is particularly true of the group of five animals representing the period of forty-eight to sixty hours. This difference appears to be dependent in part upon the extent of necrosis and in part upon the general condition and age of the animal. It has been observed, not infrequently, in animals not included in this series that when extensive necrosis of the liver was associated with severe general disturbances, indicated by marked hemoglobinuria and exhaustion, no repair was evident even in those animals surviving to the end of the second day. These various influences have been emphasized by Podwyssozki, who found that mitoses are most abundant when the conditions of nutrition are best. Thus he found mitosis to be delayed in starved animals, after considerable loss of blood and in old animals. In the latter they may fail to appear, as may they also in the neighborhood of suppuration. They are very seldom seen at the margin of areas of complete necrosis.

The primary necrotic lesion may be described as follows: After twenty-four to forty-eight hours the liver presents a uniformly mottled appearance; fine, irregular, yellowish brown or grayish yellow, non-elevated areas being sharply separated from deeply congested or occasionally even hemorrhagic areas. On section the brownish portions have a distinct hyaline appearance. The superficial portions of the liver are uniformly more extensively involved than are the deeper portions, though in some of the small lobes the necrosis may be quite general. Histologically, the necrosis, which is hyaline in character, may involve all portions of the liver tissue except circular areas of varying size in the immediate neighborhood of the larger portal spaces.

In the necrotic areas the destruction, as far as the hepatic cells are concerned, is uniform and complete; the cells of the capillaries may, however, persist. A narrow ring of liver cells with vacuolated protoplasm and pyknotic nuclei separates, as a rule, the necrotic from the normal tissue. Leucocytic infiltration may or may not be present. The capillaries of the necrotic tissue are widely dilated and tightly packed with swollen and distorted red blood corpuscles which may occur in masses or may retain distinct outlines. In the congested portal veins, on the other hand, the fusion is quite constant, the plugs for the greater part having a distinctly hyaline appearance. The perivascular spaces of the capillaries are distended with serum, and the bile passages of all sizes are, as a rule, dilated and engorged with bile.

The earliest evidence of repair, as indicated by karyokinesis of parenchymatous cells, is seen in the liver of an animal which died exactly thirty-eight hours after injection. The necrosis in this instance is very diffuse and accompanied by slight leucocytic infiltration. The destruction of liver as well as endothelial cells in the affected areas is complete. Between the completely necrotic cells and the normal cells is seen a rim of cells two or three deep in which the cell structure is more or less preserved; in these the nuclei are visible but the cell protoplasm is very granular and contains many vacuoles. It is noteworthy that mitoses are not found in such cells but only in the well preserved cells beyond (see Fig. 1). They are very few in number and are found only after prolonged search. This agrees with Podwyssozki's observation that mitoses occur only in perfect cells at a distance from the necrosis. There is some evidence, however, as will be shown later, that the nuclei of apparently degenerated cells, at the edge of the necrosis, may divide by direct division to form multinucleated protoplasmic masses. There is no evidence at this period of proliferation of the endothelial or connective tissue cells or of the cells of the bile ducts

The period from forty-eight to sixty hours is represented by material from five animals. In these the repair is of the same general character but differs somewhat in degree.

Aside from the mitoses of liver cells, which are more abundant, the most striking change is the proliferation of the cells of the supporting framework. The necrotic foci are, on all sides, separated more or less from the normal liver cells by a zone of closely massed newly-formed endothelial cells (see Fig. 2). Proliferation of the same cells occurs in the persisting capillary framework in the midst of the necrosis. The nuclei of these cells, although distinctly vesicular in character, are rich in chromatin and stain deeply. The protoplasm as a rule cannot be distinguished but it may be seen as a faint ring; when distinctly evident it takes a bluish tinge with hematoxylin. Although mitotic figures in such cells are seldom seen the above characteristics indicate a rapid proliferation. Not infrequently at the periphery of the lobule several of these newly-formed endothelial cells surround, in a ring-like manner, hyaline fragments of necrotic cells and simulate in appearance multinucleated cells into which, as will be shown later, many of the cells develop. Occasionally a stray leucocyte is seen in a necrotic cell. Phagocytic activity on the part of single cells within the necrotic focus is indicated by the occasional inclusion of a red blood corpuscle or fragments of necrotic liver cells. A very definite phagocytosis is also evident in the endothelial cells and especially in the Kupffer cells of the capillaries in the immediately adjacent normal liver. Here ten or twenty compressed and poorly staining blood corpuscles may be seen in a single endothelial cell. It is possible that some of these cells may have migrated from the spleen, where they are also very abundant, but the majority are definitely attached to the walls of the interlobular capillaries. In the endothelial cells definite mitotic figures, which can be readily distinguished from those in liver cells, are occasionally seen. They are, however, in proportion to the amount of proliferation, very few in number. Mitoses in liver cells are easily found, but always at a distance from the necrosis. They are never found in the vacuolated cells at the periphery, though between such the proliferation of endothelial cells is most active.

The picture of the third day is that of more advanced replacement of the necrotic cells by young endothelial and connective tissue cells. In many small areas these cells have more or less obscured the hyaline fragments of liver cells. Mitotic figures are more numerous in both liver and endothelial cells and for the first time are seen the large, pale oval or round liver cells described by Podwyssozki, Ponfick, and v. Meister. There is also evident an increase of chromatin in the nuclei of the liver cells. This occurs as a stippling due to numerous closely packed dots and granules which stain deeply and is suggestive of a premitotic activity.

During the fourth, fifth, sixth, and seventh days, the repair progresses with great rapidity but shows few new features. The bulk of the necrotic tissue is gradually replaced by the rapidly proliferating cells. Here and there, however, a few isolated necrotic hyaline masses, usually surrounded by very definite multinucleated cells of endothelial origin, may be seen. Mitotic figures in liver, endothelial and connective tissue cells are readily recognized; in the liver cells they are very abundant, three or four occurring sometimes in a single field of a high power lens. This rapid formation of liver cells occurs not only at the periphery but may, by the fourth day, be seen also in the midst of the destroyed area. Intercellular fibrillæ in the new tissue, first recognizable on the fourth day, rapidly increase and are very definite by the seventh day. Lymphoid cell infiltration first becomes prominent at this period and persists to a moderate extent in the later lesions. Such infiltration, however, is more abundant in the portal spaces, where there is also a proliferation of connective tissue, than in the repair about the necrotic areas. The leucocytic infiltration remains slight.

Despite the most careful study it has been impossible at this stage to determine anything definite concerning the proliferation of the small bile ducts. Not infrequently cells arranged in a circular or longitudinal manner and closely resembling newly-formed bile ducts have been seen. These have no relation to adjacent bile channels and no definite lumen. They closely resemble, however, the processes which

Marchand described in yellow atrophy as arising from isolated groups of liver cells and assuming the appearance of pseudobile ducts. That they are such appears to be very probable, but absolute demonstration is lacking as it is not possible, even in serial sections, to differentiate in the closely packed mass of proliferating endothelial and connective tissue cells between them and other newly-formed structures. As will be shown later, however, the proliferation described by Marchand can be definitely demonstrated in the more advanced repair and it is possible that the ill-defined structures here described (fifth day) represent the earliest stages of this process.

Of the lesions beyond the seventh day, some on account of the small areas involved in the primary necrosis are unfortunately of little value in studying the intermediate stages of repair. In all, however, there is a very clear picture of the gradual transformation of the young granulation tissue into a very definite connective tissue and of the slow replacement of the latter tissue by newly-formed liver cells but no new features are presented. In one (eleventh day) endothelial cells in which red blood cells are frequently seen are very numerous; in another (twenty-fifth day) lymphoid and plasma cells, usually present in but small numbers, are very abundant in and about the portal spaces.

The lesions of these intermediate stages, as well as the minute necroses of earlier periods, have been studied carefully to determine whether small areas of necrosis may be repaired by proliferation of liver cells alone without the formation of new connective tissue. Without going into detail it may be stated that such repair, except in minute areas involving but a few cells, has not been noted. In small areas the new formation of liver cells may be so abundant that the appearance may be that of simple hyperplasia of the parenchyma, but by careful study and the use of special stains a new formation of the supporting tissue above that necessary for simple restitution can always be determined. There is, on the other hand, very definite evidence, based upon a comparative study of late lesions of varying extent, that the superfluous connective tissue may be reduced to a minimum when the full development of the new liver cells is reached. Moreover, it can be definitely stated that in the smaller necroses a new formation of bile ducts does not occur; such proliferation takes place apparently only after the destruction of large areas of liver tissue.

The most advanced stage of repair occurring in this series was seen in an animal killed on the thirty-sixth day. The condition present, essentially a cirrhosis, may be described in a general way as a replacement of very extensive necrotic areas by broad bands of very vascular connective tissue. In and about the new tissue important changes in the liver cells, as well as an excessive formation of new bile ducts, are seen. The liver cells outside of the zone of new tissue are compactly arranged, often irregular in shape and, at the extreme edge of the reactive process, even misshapen. The majority stain normally but some are swollen and pale with a stippling of the nucleus. Mitotic figures may still be seen in such cells but they are widely scattered. Between these more nearly normal liver cells and the new fibrous tissue is a narrow margin in which fragments of necrotic liver cells still persist. Into this zone from the periphery project large multinucleated liver cells very irregular in shape. Similar cells, with oftentimes ten to fifteen deeply staining nuclei, surround fragments of necrotic cells. Some of these giant cells are in continuity with the strands of normal liver cells while others are entirely isolated. Such cells may contain four or five hyaline particles which they apparently englobe and disintegrate. These multinucleated masses are essentially foreign body giant cells and though a few of the smaller cells are certainly of endothelial origin the majority appear to be altered liver cells. The protoplasm, which is large in amount, is characteristic of that of the liver cell in every way and, moreover, these cells not infrequently may be seen in direct continuity with strands of liver cells of normal appearance. Isolated giant cells of similar structure but without inclusions are also seen. The material enclosed is in greater part hyaline necrotic fragments of liver cells, but consists also, as determined by micro-chemical tests, of calcified material, occurring as smooth, almost colorless disks. Between these cells a fine connective tissue penetrates but the great bulk of this zone is made up of giant cells. Similar cells are also seen in the midst of the denser fibrous tissue, sometimes occurring singly, but more frequently in connection with isolated islands of liver tissue.

A careful study of these cells leads to but one conclusion. They are altered liver cells which have developed as the result of an irregular type of nuclear division and which have acquired phagocytic powers which allow them to englobe and destroy necrotic material. There is a possibility that they may be formed by the fusion of several liver cells surrounding necrotic particles, but the occurrence of isolated multinucleated cells without inclusions indicate that such an explanation is not applicable to all these masses (see Fig. 3).

These cells are entirely different from the multinucleated structures formed by the advancing sprout of a new bile as described by Podwyssozki and have not, as far as I can determine, been previously described. McPhedran and MacCallum,[15] in their account of the histology of acute yellow atrophy in man, picture somewhat similar structures but in their description refer to them as cells of the bile ducts. They also describe both endothelial and hepatic cells containing inclusions, but not the multinucleated type of liver cell about necrotic fragments.

Proliferation of the bile ducts while most evident in the new tissue is seen also in the adjacent normal liver. In the latter location, from the canals of medium size, a solid process of newly-formed cells may not infrequently force its way between the normal liver cells and penetrate the zone of multinucleated cells. Oftentimes these strands, which are never canalized, show a division into terminal sprouts composed of conglomerate masses of cells resembling in a rough way giant cells.

Within the new connective tissue new bile ducts are very numerous. In serial sections these may sometimes be seen to have a connection with normal ducts but more frequently they have no such connection and appear to be the results of the proliferation of persisting fragments of canals which had escaped the primary necrosis. For the most part they are solid processes with irregular swollen branchings or sprouts. Many of these budding processes have on cross section, as has been noted by Podwyssozki, the appearance of giant cells.

Those which have a distinct lumen are usually widely dilated and are either empty or contain a few leucocytes or desquamated cells. Such are usually irregular and tortuous with one or more solid branching processes. In form and structure the cells of which they are composed are intermediate between the true liver cell and the epithelium of the normal bile duct (see Fig. 4). They are usually irregular, or polyhedral in shape, though often oval but never cylindrical. They have a large amount of protoplasm which tends to stain lightly with eosin and closely resembles that of the liver cell. In the solid masses the protoplasm is not so evident. The nuclei are round or oval and, as a rule, vesicular. That these structures are truly newly-formed is shown by the presence of mitotic figures which though not numerous may be found without great difficulty. In the older portions of the solid processes, as well as in the distinctly tubular structures, not infrequently may be found single cells much larger than the others and with protoplasm very similar to that of the fully developed liver cell. Similar cells of varying size may also be seen in the adjacent connective tissue either as isolated cells or, more rarely, attached to the new bile duct by a thin thread of protoplasm. Such pictures indicate conclusively that liver cells are formed from these structures. The formation of new liver cells from the terminal budding process I have not observed though it is very probable, as Podwyssozki states, that they may eventually become isolated and be transformed into liver cells. At the stage here studied such transformation has, however, not been seen.

In addition to these processes, concerning the origin of which from preëxisting bile ducts there can be no question, other cell strands of very similar structure which are formed with equal certainty by a proliferation of liver cells are occasionally seen. These are always connected, with the normal liver substance at the periphery or with small islands of liver cells persisting in the center of the new tissue. They occur as closely massed double rows of cells or as groups of cells, usually with budding processes, but never canalized. The cells of which they are composed are similar to those of the solid processes arising from persisting bile ducts and from such processes they cannot be distinguished in their terminal portions; but upon tracing them to their origin they are found to be connected with true liver cells. The transition of the liver cells is gradual and all stages can be traced (see Fig. 3). The point of most abrupt change is usually at the narrowest part of the strand. That these structures may later become canalized is possible but it seems more probable that they represent rapidly growing cells which later develop into typical liver cells. Such transformation, however, has not been observed. That these strands are not the result of compression of liver cells is shown by the fact that the most active growth is in the distal portion and in the midst, as a rule, of necrotic cells or newly-formed fibrous tissue. These structures are apparently very similar to those seen by Marchand in acute yellow atrophy of the liver.

SUMMARY.

In the necroses of the dog's liver, due to the injection of hemagglutinative sera, the first evidence of repair is the occurrence after thirty-eight hours of mitotic figures in the liver cells. At the end of the second day these have greatly increased and a considerable proliferation of endothelial cells has occurred in and about the necrotic areas. These last tend to encapsulate the necrotic cell fragments. From the third to the seventh day these processes advance, and with the formation of a young granulation tissue, the necrotic areas are largely replaced. While there is some evidence that areas of necrosis involving only a few cells may be repaired by proliferation of liver cells, without the formation of connective tissue, in all areas of appreciable size the latter is a prominent feature. Later, however, as the liver cells increase they replace the connective tissue to great extent. The coarser bands, however, remain. Throughout the entire process of repair the great power of the liver cells to increase by indirect division is the most striking feature. In the late stages of the repair of extensive necrosis many multinucleated liver cells, most of which surround fragments of necrotic cells, may be seen between the normal liver and the dense bands of new tissue. Here are seen also numerous giant liver cells without inclusions. New bile ducts formed from the uninjured larger ducts or from persisting fragments of the smaller canals, aid, by a transformation of their cells into liver cells, in the restitution of the liver parenchyma. The formation of new ducts and the transformation of their epithelium into liver cells occurs, however, only when large areas of liver substances are destroyed. Other cell strands, of the general appearance of new bile ducts, are formed to a slight extent by the direct proliferation of liver cells.

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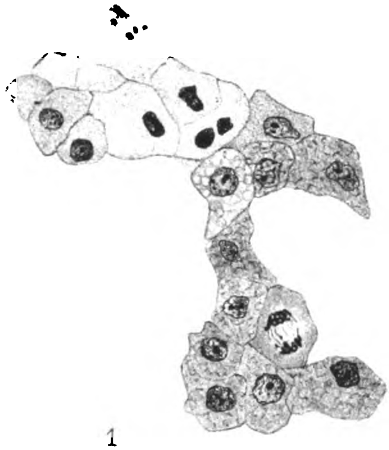
EXPLANATION OF PLATE XIV.

FIG. 1. Mitotic figure in a liver cell in the neighborhood of a necrotic area. Thirty-eight hours. 4 oc., 1/12 obj., Leitz.

FIG. 2. Edge of necrotic area showing proliferation of endothelial cells and tendency of latter to encapsulate necrotic cell fragments. In the center is a necrotic cell containing a polymorphonuclear leucocyte. Sixty hours. 4 oc., 7 obj., Leitz.

FIG. 3. Solid process of cells formed by proliferation of liver cells and resembling a branching non-canalized newly-formed bile duct. Below is a multinucleated liver cell inclosing necrotic cell fragments; above is a similar giant cell without inclusions. Thirty-six days. 4 oc., 7 obj., Leitz.

FIG. 4. A newly-formed bile duct in which may be seen a mitotic figure and cells of a type intermediate between true liver cells and the epithelium of the normal bile duct. Thirty-six days. 4 oc., 1/12 obj., Leitz.



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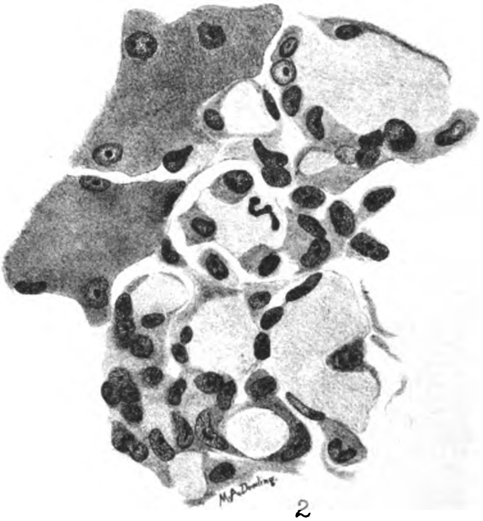

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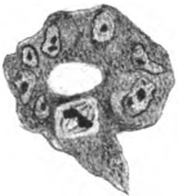

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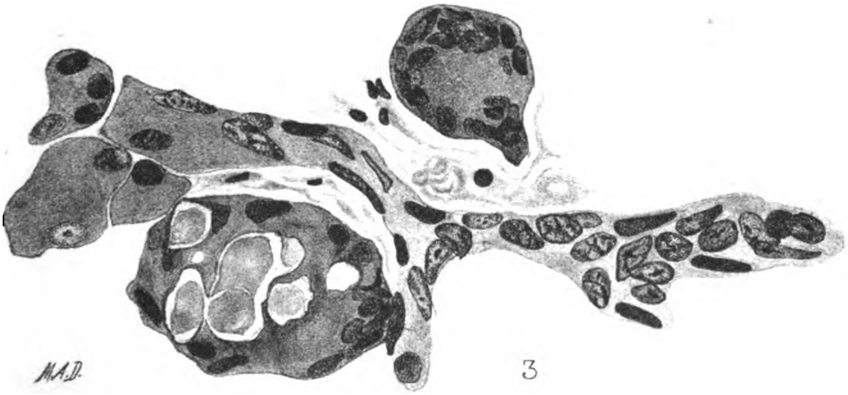

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Pearce.

Regeneration of the liver.

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A SIMPLE WAY OF USING BUCHNER'S METHOD FOR THE CULTIVATION OF ANAËROBIC BACTERIA.*

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In 1888, Buchner introduced the pyrogallic acid method for the cultivation of anaërobic bacteria and described what he called the simplest arrangement for the use of this method, namely, the double tube arrangement. While the principle involved in Buchner's method has been adopted unchanged, several different ways of applying it have been devised in the interest of further ease and simplicity. Thus, for dealing with cultures in test-tubes, the ordinary preserving jar has been substituted for Buchner's outer tube, and Wright has introduced the practice of impregnating the plug with the alkaline pyrogallic solution and closing the tube with a rubber stopper. The apparatus here described, which was suggested by Dr. James Carroll, is the result of another effort in the same direction.

The apparatus consists of (Plate XIV. A.):

A. The culture tube and a dry empty test-tube of equal diameter.

B. A short U tube with a diameter equal to that of the test-tubes.

C. Two pieces of rubber tubing for making connections.

After inoculating the culture tube, the plug is pushed in a little below the lip of the tube; the ends of the U tube and test-tubes are coated externally with vaseline, the rubber tubes are adjusted on the U tube and a connection made with the culture tube so that the glass ends meet. One or two grams of pyrogallic acid are put in the empty test-tube and packed down with a little filter paper over it; ten or twenty cubic centimeters, respectively, of a ten per cent solution of sodium hydroxide are then poured into the tube and

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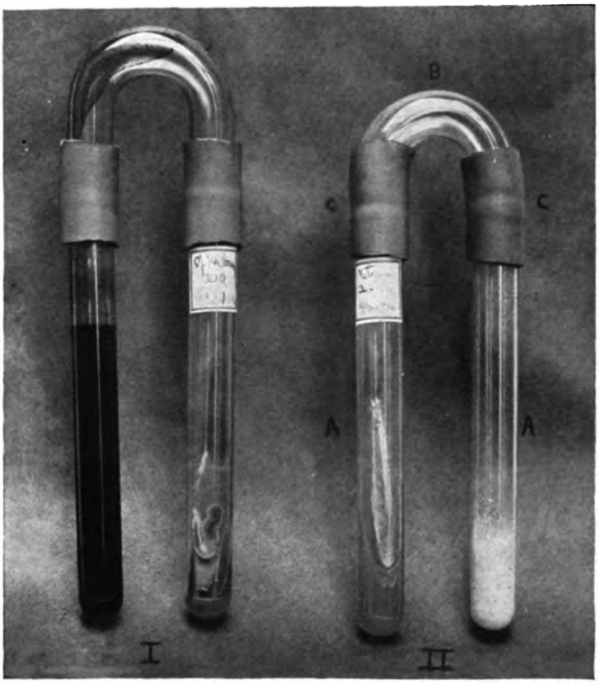
the second connection is made quickly before the acid and alkali react to any extent.

This procedure can, of course, be applied to cultures in any of the ordinary media in test-tubes, including Esmarch rolls, and also to cultures in fermentation tubes. Aside from its simplicity, its advantages are that very little oxygen from the outside of the system is absorbed before closure; that comparatively little oxygen must be absorbed from within the system; that the culture tube is not soiled inside or out by the reducing solution; and more particularly that the growth in the media can be directly observed without opening the apparatus. This is a matter of some importance in surface growths of such organisms as Bacillus tetani, which at best grows but faintly on the surface. Its only disadvantages would seem to be the occasional difficulty of procuring or making U tubes of the proper size and the necessity of using one for each culture tube.

The absorption of oxygen was tested in a way similar to that originally used by Buchner, and was found to be complete in twenty-four hours. Culture tests were made chiefly with Bacillus tetani. Stab cultures in deep glucose agar showed growth with lateral offshoots extending clear to the surface. Glucose bouillon became turbid in three to four days. Surface growth on glucose agar became visible in five days. The behavior of milk was especially interesting. The litmus gradually lost its color and in a week the medium was entirely white without coagulation. On opening the apparatus the upper part of the milk immediately became pink, and in a few hours all the milk became blue again. This behavior has not been noticed with other organisms.

Several variations were tried of combining the acid and alkali so that no outside oxygen would be absorbed; the acid was added to the alkaline solution in a gelatine capsule; the alkali, in stick form, was added to a watery solution of the acid, but the simple procedure given above seems the most satisfactory. The reduction of the size of the plug of the culture tube by teasing out and flaming was also tried, but this, too, seems unnecessary. At first no U tube was used and the tubes were connected directly by rubber tubing. This arrangement, however, is not firm, and the tubing partly collapses, becomes kinked and may also be somewhat permeable to oxygen. No determination was made of the exact amount of acid and alkali necessary to absorb the oxygen from the system. Buchner figured on one gram of both acid and alkali to exhaust one hundred cubic centimeters of air; this apparatus contains only about fifty cubic centimeters of air, but it seems advisable to use about two grams of each ingredient to be on the side of safety.



I. SPIRILLUM RUBRUM. — Glucose agar slant culture of five days. Abundant production of pigment on the surface. The U-tube was soiled by the reducing fluid during handling by the photographer.)

II. BACILLUS TETANI. — Alkaline glucose agar slant culture. Surface growth of twenty days. In the photograph the growth shows more distinctly than it does in the culture tube.

The second tube shows the pyrogallic acid packed down ready for the solution of sodium hydroxide. It is placed there for the purpose of illustration only, the original, containing the fluid mixture, having been removed after the development of the growth.

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TRYPANOSOMATA AND TRYPANOSOMIASIS.*

(*A Summary.*)

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Definition. — Trypanosomata are flagellate Protozoa causing a specific disease, Trypanosomiasis, in man and many of the lower Vertebrata.

History. — Valentine (Berne) discovered in 1841 the first trypanosoma. He observed it in the blood of the trout (*Salmo fario*). In 1842 and 1843 Gluge (Brussels), Mayer (Bonn), Gruby (Paris), published three works on the trypanosomata of the frog. It is for this organism that Gruby created the name Trypanosoma (τρύπανον, auger; σῶμα, body), the Greek plural for which would be Trypanosomata. Wedl, 1850, Chaussat, 1850, Ray-Lancaster, 1871, Rattig, 1875, described the parasite in the blood of Batrachians. Remark, 1842, Gros, 1845, Berg, 1845, Chaussat, 1850, described the organism in fishes. Gros in 1845 found it in the blood of the field mouse and of the mole. In 1850 Chaussat saw it in the blood of a black rat, but it was not until the work of Lewis in 1878 on the parasites in the rats of India that attention was really drawn to the trypanosomata of mammals. In 1880 Evans discovered trypanosomata in the blood of horses suffering with the well-known surra of India. He proved the parasite to be the cause of the disease. Wittich, 1881, discovered a trypanosoma in the blood of hamsters. Marchiafava and Celli in 1885 found a trypanosoma in the blood of a patient ill with malarial fever. Rouget, 1896, described the organism in the blood of a horse ill with dourine, though Voges says Chauvrat discovered it in 1892. Bruce described the organism of Nagana in 1897. Elmassian in 1901 first differentiated the trypanosomata of mal-de-caderas in South America. Theiler, in

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an article published by Bruce, is credited with the discovery of a new trypanosoma of cattle in South Africa.

CLASSIFICATION.

Trypanosomata belong to kingdom Animalia, sub-kingdom Protozoa, class Mastigophora, sub-class Flagellata, order Monadida, family Trypanosomidæ, genus Trypanosoma.

Class; Mastigophora, Butschli. — Class diagnosis — Protozoa. Microscopic organisms, whose bodies usually have a definite form, but are not always provided with a cell membrane; cilia are absent, but the adult stage possesses one or several flagella; mouth present or absent; usually only one nucleus, but never a paranucleus; usually one or several pulsating vacuoles; reproduction by division, with or without conjugation and encystation.

The systemic position and limits of this class are in doubt. They may be placed under rhizopods or near the ciliates. Some of the forms which certain authors admit to the class as animals others call plants. Some described as flagellates may be only developmental stages in the life cycle of other animals, as flagellate stages occur in the life cycle of some of the Mycetozoa and Sporozoa. Some species are exceedingly simple in character, others are much more complicated and give rise to more or less complex colonies.

Sub-classes of the Mastigophora. — 1. *Flagellata.* There is always a flagellum at the anterior end and frequently two, and others may be present on other parts of the body.

2. *Choanoflagellata* (Gr. χοάνη, a funnel), readily distinguished by the presence of a protoplasmic collar or cup, which surrounds the base of the flagellum, and both the collar and flagellum are contractile.

3. *Dinoflagellata* (Gr. δίνος, a whirl) generally possess two flagella, one extending freely from the anterior end of the body, the other lying in a groove which passes around the body transversely.

4. *Cystoflagellata* are marine forms having a cyst-like structure.

FLAGELLATA.

These are small organisms, usually with sharply defined mononuclear body, with a definite anterior end bearing one or more flagella; actively motile during life, but possessing power of encystment; reproduction by longitudinal division in free or encysted stage.

Flagellata represent the only sub-class of Mastigophora which at present comes into consideration as parasites. Owing to the minuteness of the objects and the difficulty of recognizing characters for classification, there is a wide difference of opinion among authors relative to the sub-division of the sub-class into orders, families, genera, and species.

Order: Monadida.—They are independently living organisms, in some cases forming colonies; arrangement of the flagella varies; one flagellum, usually anterior, or two equal flagella, or two unequal flagella, or one large and two small flagella; undulating membrane may be present.

Family: Trypanosomidæ, Doflein, 1901.—They are parasitic forms with one chief flagellum, directed anteriorly; in some forms a secondary flagellum directed posteriorly; body usually with two angles, and wound more or less in the form of a spiral, one angle of body provided with an undulating membrane. One nucleus and one centrosome present.

The family at present contains two genera which are distinguished as follows:

1. *Trypanosoma*, one flagellum, extending from the centrosome along the undulating membrane and becoming free at the anterior extremity.
2. *Trypanoplasma*, two flagella, one extending anteriorly, the other posteriorly.

GENUS TRYPANOSOMA, GRUBY, 1843.

The body is fusiform, presenting a lateral longitudinal undulating membrane, the thickened border of which terminates posteriorly in the posterior half of the body, in a centrosome, and is prolonged anteriorly in a free major flagellum; nucleus generally anterior; there is a tendency to agglomeration by the posterior extremity, division longitudinal and unequal. It is parasitic in the blood of animals. Type species Trypanosoma rotatorium.

General characters. — The organisms all show very active eel-like movements and some motility. In some species the latter is slight, the parasites undulating with extreme rapidity, but covering so short a distance as to be easily followed under the microscope; while in others, especially Tr. Lewisi, the movements are so rapid that it is impossible to keep the parasite in the field. The flagellum at the anterior end varies greatly in length. It is always actively motile, pointed, and continuous with the thickened margin of the undulating membrane, ending at or near the centrosome. It may be entirely homogeneous, or it may contain from one to several distinct granules, extending well out from the body of the parasite. The undulating extends along one border of the organism from the centrosome in the posterior end to the anterior end, whence it continues as the free flagellum. Its folds and breadth vary with different species and with the age of the organism. The centrosome is at the posterior end and appears to have intimate associations with the flagellum and undulating membrane. Laveran and Mesnil say it is the center for external movements. Its varying distance from the posterior end has been used as a diagnostic point in determining species; but not much importance can be attached to this, for it has been shown that the posterior end of the organism is contractile. The protoplasm is homogeneous or granular depending on age, environment, and species. The granules may vary in number and size from a few small ones situated in the anterior portion to numerous large ones scattered through the protoplasm. The nucleus is usually in the anterior half of the parasite, and is generally round or oval. Trypanosomata are nourished through osmosis. Neither digestive vacuoles nor solid inclusions of any sort are found in them. They displace by their movements the red corpuscles. But they have never been seen to attack them, either to surround them or take them within. McNeal and Novy have shown that hemoglobin is necessary for their growth.

Reproduction in flagellates generally takes place by simple division, either during the active condition of the individual, or after it has secreted a cyst about itself; in the latter case a division may result in two, but often in several new individuals. In the colonial Flagellata each individual in some genera divides a number of times and forms a colony. In some Flagellata there is a more complex method of reproduction which closely resembles the sexual. Some cells enlarge and become macrospores, others divide a large number of times and become microspores; these two kinds of spores fuse with one another, and then division results in a new colony.

Rabinovitsch and Kempner describe three modes of multiplication in Trypanosoma lewisi: longitudinal division, transverse division, and segmentation. Wasielewski and Senn by study of living preparations, and by employing an improved staining method, were able to show that the differences in arrangement of nuclei of the dividing cells, which was for the former workers a characteristic distinction between longitudinal and transverse division, were due to manipulation. They further showed that the characteristic feature of segmentation, namely, that division was initiated only after loss of the undulating membrane and flagellum, and rounding up of the parasite did not exist in reality. They thus established unequal longitudinal division as the only method of multiplication. This is confirmed by McNeal.

Prowazek announces in a few lines the observation of conjugation in Tr. Brucei without giving any description of the process. McNeal has found couples in such intimate contact that a magnification of x 3,000 fails to find any line of separation. Exchange of material from one cell to another has not been demonstrated, however, and the question is not yet settled.

Trypanosomata are found in all the body fluids, and are not present at the same time in enormous numbers in one part with but few in another. Animals having many parasites in the blood when killed show them also in the organs. The blood of animals suffering from the disease is always infectious by animal inoculation, although there are periods during its course when the parasites cannot be found for days by microscopic examination. McNeal and Novy have found that cultural methods will disclose the organism when microscopic examination has failed. Plimmer and Bradford and others state that the lymphatics near the point of inoculation first show the parasite, and that animal's blood may be infected for two days before the parasites are found in it. Martini regards the spleen, lymphatics, bone marrow, and to a less extent, the liver and kidneys, as the places for the destruction of the organism. Experiments to determine the place of multiplication tend to show the whole cycle takes place in the blood. Parasites live only a short time in the body after death. Within two hours signs of degeneration begin; the parasites shrink, assume irregular shapes and then disappear. Motile parasites are not usually found two hours post-mortem. In exceptional cases living parasites have been found as late as sixteen hours after death. After twenty-four hours — at longest forty-eight hours — the blood is not infectious. Trypanosomata are usually more numerous in the spleen, liver, and lymphatic glands than in the bone marrow, and are seldom present in the medullary canal. They are found in the serous fluids and exudates of the joints, but rarely in the urine.

Trypanosomata may be examined in the fresh state by taking a drop of blood from the infected animal; in mammals, the ear; in rats, the tail; in birds, the great vein on the inside of the wing; in reptiles, from the tail or toe; in batrachians, from the fore foot; and in fishes, from the tail fin. It is often necessary to examine many preparations and sometimes for several successive days. Kanthack, Durham, and Blandford have advised centrifugalizing the defibrinated blood. The trypanosomata are found in the middle layer with leucocytes. In dourine the examination can be made from the bloody edematous fluid, and in sleeping sickness the blood and the cerebro-spinal fluid from a lumbar puncture should be examined. As trypanosomata are rare in this fluid, it should be centrifugalized before examination. In prolonged observations a hanging drop may be used, sealed with paraffin or vaseline. This is useful for studying the phenomenon of agglutination. The blood is mixed with physiological salt solution or physiological citrated solution to prevent coagulation, or with the serum of another animal. Francis advises, for Trypanosoma lewisi, letting the blood coagulate, the trypanosomata pass into the serum and can be studied free from blood corpuscles. After an hour the motility of the organism quiets down. To check the active movements, Plimmer and Bradford advise a drop of a 1–100 solution of gelatine to the drop of blood.

The organisms are stained with eosin and methylene blue by various methods. The most common and best is the Romanowsky or some of its modifications. Wright's modification of the Romanowsky's stain is used, but it does not bring out the eosin staining parts well. Wright's stain made with commercial wood alcohol as used by Wolbach is the best stain. It brings out the structure of the undulating membrane as no other stain does.

Laveran and Mesnil found that Tr. Lewisi died out in defibrinated blood in four or five days, while if placed in an ice-box at 5° C. to 7° C. they remain alive for fifty-two days. At —17° C. they die out in two hours, and the blood fails to infect animals. Hanging drops and capillary tubes kept at 5° C. to 10° C. for thirty-two to fifty-three days infected rats after a period of seven days. Tr. Brucei may be kept alive in the blood for two to three days, whereas in the dead body they die out in twenty-four hours. Laveran and Mesnil say blood mixed with physiological salt solution can be kept in a virulent state for three days at room temperature. In undiluted blood they become inert in forty-eight hours, whereas if diluted with horse serum the parasites could be kept alive for three days. When kept in an ice-box at 5° C. and 7° C. they die in three days. When the blood is subjected to —50° for one-half to two hours and then melted, the organisms were still alive. Rouget says the trypanosoma of dourine in fresh blood preparations was motile for eighteen hours, but never after twenty-four hours. McNeal and Novy found that trypanosomata could be kept alive for several months when the infected blood was added to rat, rabbit, or guinea-pig blood. Bacteria soon destroy the organism, probably by altering the medium and more especially the hemoglobin. The fact that the trypanosomata lived in the rat blood when diluted with other blood for a considerable period, even at ordinary room temperature, whereas in undiluted blood they die out quite early, indicated that possibly multiplication took place.

Danilewsky is usually credited with having cultivated trypanosomata in capillary tubes, but this is not substantiated by any statement of his own. According to Rouget, Chalachkow is credited with growing the parasite on dog serum. Six days after inoculation he observed, besides the ordinary forms, new ones showing different degrees of development. McNeal and Novy were the first to use cultural methods successfully. They added sterile defibrinated blood to ordinary nutrient agar. For this purpose the agar was melted and then cooled to 50° C., when it was mixed with sterile defibrinated blood, usually two parts to one, after which the tube was slanted and allowed to solidify. The medium thus prepared was bright and contains unaltered hemoglobin. The latter appears to be essential, for when the blood agar was kept in the incubator the hemoglobin underwent a change, and it was no longer a useful medium.

The blood was usually drawn from the carotids into sterile pipettes. A glass rod was passed through the cotton plug before sterilizing and by means of this the blood was readily defibrinated. The transfer of the defibrinated blood to the melted agar was made with sterile Pasteur bulb pipettes. In order to secure from the rat the largest possible yield of trypanosomatous blood without bacterial contamination, the best procedure is to draw it directly from the heart into a sterile pipette.

With reasonable care blood agar can be thus prepared without the least danger of bacterial contamination. As soon as the blood agar cools and becomes solidified the tube is placed upright so as to allow as much water of condensation as possible to collect. This water is then inoculated with the otherwise sterile trypanosomic blood. When the inoculated tubes are to be kept at room temperature for weeks and months, it is necessary to prevent desiccation. This can be done best by sealing wax — rubber caps are not so useful. In culture experiments at incubator temperature, a different procedure was found necessary. The tightly rolled cotton plugs were first cut short, then charred in a flame, and pushed inside the tube. A number of these were then placed in a large desiccator, or preferably in a Novy anaërobic plate apparatus, on the bottom of which was some cotton well soaked in mercuric chloride. In this way an abundance of moisture and oxygen was provided for, and at the same time desiccation of the tubes prevented. By wiping down the outside of the tubes with mercury solution practically all danger of mould contamination is avoided. The growth at incubator temperature is more rapid, reaching a maximum in eight to twelve days, and death takes place in three weeks. Cultures at room temperature retain their vitality for weeks and months. Rat trypanosomata will grow on blood agar of 1 to 2 or 1 to 5 or even 1 to 10. The best conditions obtain with a 2 to 1 blood agar.

Culture of Tr. Brucei. — Tr. Brucei will only exceptionally grow on a medium which contains less than one-half its volume of blood. Sometimes only one or two tubes will develop out of a large number, although the medium is the same in all the tubes. For isolation from the living animals a nutrient agar of the following composition is prepared:

Extractives of 125 grams chopped beef in distilled water .	1,000 cc.
Agar	20 g.

Peptone	20 g.
Common salt	5 g.
Normal Na_2Co_3 solution	10 cc.

The agar thus prepared is distributed into tubes or small Erlenmeyer flasks and sterilized in the autoclave. To one volume of the sterile melted agar, cooled to about 60° C., two volumes of naturally sterile defibrinated rabbit's blood is added; thoroughly mixed and allowed to solidify. Tubes are allowed to solidify in the inclined position; flasks upright. Into the small amount of liquid which collects on the surface, pure Nagana blood is inoculated, and here development takes place.

Culture of Tr. Evansi. — The medium employed for the cultivation of Tr. Evansi was the same as that employed for the cultivation of Tr. Lewisi and Tr. brucei. Tubes inoculated January first in the Philippines were kept in cold storage three days and then shipped to Ann Arbor, where they arrived February eighth, or thirty-eight days later. There was a good growth, but all attempts at inoculating animals or obtaining subcultures failed. This is due to the fact that the organisms were thirty-eight days old when received.

Culture of Trypanosomata of birds. — The method employed was essentially the same as that used in Tr. Brucei and Tr. Evansi. In the work on Tr. Brucei, only eight per cent of infected animals gave cultures of this organism. This is due in large part to the concentration of meat extract used in the preparation of agar. An excess of meat extract inhibits, possibly by over stimulation, the development of the initial culture, whereas a smaller amount favors growth. After the growth is once obtained the media is the same as for Tr. Brucei.

Agar cooled to about 50° C., with two volumes of defibrinated rabbits added, is used. The water of condensation is inoculated with a drop of blood taken from the heart of the bird by means of a drawn-out tube pipette. In some instances, as when large birds are used, the blood may be drawn from the median vein by means of a sterile syringe. The cotton plug of the tube is cut short, moistened with mercuric chloride, and the tube covered with a rubber cap, after which it is placed at 25° C. for about a week. Growing trypanosomata may be recognized on the third day. On the sixth to seventh day, they are usually extremely abundant and very motile. Trypanosomata of birds differ in culture markedly from each of the three other organisms as well as among themselves. When cultivation is attempted, it is always advisable to inoculate three or four tubes of the medium for the reason that it may happen that but one out of a set of such tubes may develop.

Culture of Tr. rotatorium. — The blood of frogs and toads was taken to make blood agar. The blood was first examined to see that it was free from parasites. The animal was then etherized and placed in $HgCl_2$ solution (1–1,000) for fifteen minutes, rinsed in distilled water, opened with all precautions, the blood from the heart taken with a sterile pipette, and mixed rapidly with water of condensation on slanted agar tubes (made with meat extract, peptone, and slightly alkaline to litmus). Two or three drops of blood were used for each tube. The tubes were sealed with rubber stoppers and allowed to stand five to ten days, so that contamination with bacteria might be detected.

The blood of a frog infected with Trypanosoma rotatorium collected in the same manner was mixed with that in the blood agar tubes prepared and tested as just described. The tubes were kept at room temperature. Growth took place after two weeks, and was never luxuriant. Arrangement in rosettes was never seen. One generation only of subcultures grew. All the cultures soon died. Inoculations failed.

Inoculations. — Inoculations of blood diluted with physiological salt solutions, defibrinated blood or pure blood are best made intraperitoneally. The animal must be susceptible, and the organisms must be in good condition and young. Old organisms will not infect. Young animals are more easily infected than old.

TRYPANOSOMIASIS.

Trypanosomiasis is such a widespread disease and affects so many animals that a great many names have been given it. Musgrave and Clegg give a list of eighty-two names used to designate the disease, but the list is not complete.

Symptoms. — Trypanosomiasis is usually characterized by a period of incubation, followed in most animals by a remittent, intermittent, or less frequently, relapsing fever; by the presence of trypanosomata in the circulating blood, which in some animals are in proportion to the temperature, by progressive anemia and emaciation, by catarrhal condition of the mucous membranes of the eyes and nose, by roughness of the hair, which in many instances falls out, by subcutaneous edema, more commonly of the posterior extremities, genitals, and belly. In the later stages, paresis of the posterior extremities is very common.

Pathology. — In addition to evidence of severe anemia, certain changes in the spleen are found, the most constant being enlargement and a peculiar mottling. There are also lymphatic hyperplasia, peculiar yellowish gelatinous subcutaneous and subserous infiltrations, an enlarged liver, and an accumulation of fluid in the serous cavities.

Trypanosomata Vermes. — Butschli found trypanosomata in the intestinal canal of a nematode (Tribolus gracilis). They were 33 μ in length and were sometimes observed in stellate colonies. They have also been reported in the intestinal canal of leeches (Hirudinea), having been sucked in from the blood of some higher animal.

Trypanosomata in Arthropoda. — Trypanosomata have been found in flies, gnats, lice, fleas and other Hexapods, these having obtained them from higher animals, and serving also to spread the disease.

TRYPANOSOMIASIS OF VERTEBRATES.

Pisces history. — In 1841 Valentine discovered a trypanosoma in the blood of a trout (Salmio fario). Remark, 1842, saw the organism in a pike (esoxlucius) and in many fresh water fishes. Gros in 1845 saw the parasite in a number of fishes. The list of fishes, both fresh water and marine, in which trypanosomata have been found is a long one. The Trypanosomata are rare in cartilaginous sea fish and numerous in the bony sea fish. The parasites have been described by many observers, among whom are Berg, Wedl, Chaussat, Mitrophanov, Danilewsky, Chalachnikov, Kruse, Lingnard, Sabrazes, Laveran, Mesnil, Hofer, Leger, and Plehn.

Technic. — Blood is obtained by cutting two or three rays of the caudal fin. The organisms will live in a physiological salt solution for four or five days. For stained preparations it is necessary to open the fish while still alive, take a drop of blood from the heart, dry rapidly over an alcohol lamp and fix with alcohol or alcohol and ether. Air at the sea is so humid that if the blood is left to dry the parasites become deformed. In dead fish the blood and trypanosomata are quickly deformed.

CLASSIFICATION. Trypanosoma Remaki.

Trypanosoma Remaki has a large geographical distribution and is divided into two varieties named, according to size, *parva* and *magna*.

Tr. Remaki *parva* is 28 to 30 μ long, body 15 to 20 μ. It may reach a length of 42 μ.

Tr. Remaki *magna* is 45 to 57 μ long, body 26 to 28 μ. It colors a deeper blue than the *parva* and is thicker. The structure is otherwise similar to the *parva*. These large forms are not division forms, for a single sign of division has never been seen; they may be older forms of the *parva*. When multiplication occurs in the parva, the parasite enlarges and division may begin at the nucleus, more often at the centrosome. The centrosome enlarges and divides into two, which are united by a bridge. At the same time the flagella divides at the base and then throughout its length.

Trypanosoma Danilewsky. — Danilewsky found this form in the carp (Cyprinus carpio). It is 35 to 45 μ in length, 3 μ thick. The undulating membrane is large with many folds. The protoplasm contains numerous chromatin granules of various sizes. Multiplication has never been seen.

Trypanosoma tincæ. — Doflein found it in the tench (Tinca tinca), which were sick and dying in great numbers. In fresh blood this trypanosoma is very motile and is almost always coiled up. It is about 35 μ in length. The undulating membrane is large and has many folds. Some forms show division of centrosome and commencing division of flagella.

Tr. Abramis. — Trypanosomata have been found in the bream (Abramis brama). Not much is known about this form.

Trypanosoma granulosum. — The first description of trypanosoma in the eel (Anguilla vulgaris) was that of Sabrazes and Muratet of Bordeaux. The largest forms reach from 44 to 80 μ in length. The centrosome is spherical and of good size. The undulating membrane is well expanded and bordered by a sharply defined flagella. The protoplasm is filled with granules of good size of deep violet blue. Granules are sometimes massed around the nucleus, which becomes hard to see. The nucleus is a violet red and sometimes occupies the breadth of the body, sometimes it is slender and is close to the concave side. Sabrazes kept these Trypanosoma a week at 10° C. to 19° C., in vitro and saw them multiply.

Trypanosoma soleæ. — The trypanosoma of soles (Solea vulgaris) is 40 μ long, of which the body is 32 μ and the flagella only 8 μ. The protoplasm contains some chromatic granules toward the posterior extremity, and shows some fine longitudinal striations.

Trypanosoma scylæ. — Laveran and Mesnil found a trypanosoma in the dogfish (Scylium canacula and Scylium stellare). It is from 70 to 75 μ long, the flagella 14 μ. The protoplasm is a strong blue, the undulating membrane a pale blue. The centrosome is smaller than that of Tr. Soleæ. It is very rare.

Tr. rajæ. — Trypanosomata, found by Laveran and Mesnil in Rajæ, are 75 to 80 μ long, flagella 20 μ long, about 6 μ in thickness. Multiplication forms have not been seen. It is similar to Trypanosoma Scylæ.

TRYPANOPLASMA. — Trypanoplasma are found in the Cyprinides (carp and minnows). There are two species.

Trypanoplasma Borelli. — The body of Trypanoplasma Borelli is flattened, often bent in a circle. The concave side is thick and takes a deeper color. The color of the posterior end is deep blue, while that of the anterior end is clear. It is 20 μ long and 3 μ thick. There are two masses of chromatic granules, one globular, the nucleus, on the convex side, and the other, the centrosome, on the concave side. The centrosome gives rise to two flagellæ which issue from the same pole; one runs posterior and one anterior. The trypanoplasma of the minnows is more pathogenic and has large grains of pigment, but is otherwise like Trypanoplasma Borelli.

Trypanoplasma Cyprini. — Mlle. M. Plehn found this form in two carps. This form caused an epidemic in Germany. The flagellæ are of unequal length; the anterior is more than one-half the length of the body, the posterior one-quarter the length of the body. Multiplication forms are rare.

Physiology of the Trypanoplasma. — Leger says the posterior flagellum is the helm, the anterior flagellum a feeler, and the undulating membrane the locomotive apparatus.

Methods of infection. — It is probable that trypanosomiasis of fish is spread by ectoparasites which fix themselves on the gills or on the surface of the body between the scales.

Symptoms. — Hofer describes the symptoms of the disease in the carp. The fish lie on the side for weeks, the head and tail drooping. If they are picked up, they swim around a while and then fall on the side, breathing slowly and feebly. With the weakness there is a loss of flesh.

Leger describes the symptoms in the minnow. The fish becomes pale, swells up, becomes motionless, takes no nourishment and dies. Similar symptoms have been observed by Plehn and Doflein.

AMPHIBIA. History. — Gluge in 1842 discovered the presence of trypanosomata in frogs. In 1843 Mayer and Gruby described the parasite and Gruby gave the name it now holds. Other observers are Chaussat, Wedl, Lieberkuhn, Ray, Lancaster, Rattig, Gaule, Grassi, Danilewsky, Chalachnikov, Ziemann, Laveran and Mesnil, Dutton and Todd, Ed. and Et. Sergent. Ziemann was the first to use the Romanowsky method of staining. This method gives a blue stain to protoplasm of animal organisms, and a red to the nucleus. He succeeded in staining the nucleus and centrosome, but did not stain the undulating membrane nor the flagellum.

Trypanosomata are more often found in frogs in summer, especially in August in this part of the world. The males of *Rana esculenta* have twice as many parasites as the females. Gruby says that the proportion of males infected is smaller than that of females.

Trypanosomata rotatorium. — The pleomorphism of this organism has drawn the attention of many observers. Danilewsky distinguished at least four varieties. Chalachnikov distinguished two groups. The thickened edge of the undulating membrane is in relation with a clear space, in the center of which is the nucleus. There are two varieties of Tr. rotatorium, according to Laveran and Mesnil, one with the surface covered with numerous divisions, the other flat with smooth surface. The flagellum is short in comparison with the body. The posterior end is round and blunt at times, or may be short or very long. Trypanosoma of frogs present an extreme variety of forms. It is 40 to 60 μ long and 5 to 40 μ wide. Some have an oval form, round at both ends with a short free flagellum, an undulating membrane which extends one-half the length of the body, and is 50 to 60 μ long and 30 to 40 μ wide. Multiplication forms were not seen.

Lewis and Williams, who grew cultures of trypanosomata of frogs, give the following description: Cultural forms of Tr. rotatorium show organisms of a very long oval form, the bodies of largest being 2 x 18 μ, the flagellum nearly as long as the body. Only the largest forms showed a trace of undulating membrane. Motility was not very pronounced. Numerous small forms were seen. The centrosome was at the anterior end, the nucleus at the posterior end.

Another form was 3 μ x 16 μ, flagellum hardly half as long as the body, undulating, not distinct on small forms. Large forms, similar to Tr. rotatorium, except that the flagellum was lacking, occurred, but were rare.

Trypanosomata inopinatum. — This variety measures 25 to 30 μ and 3 μ wide. It resembles very much Tr. Lewisi. It is stockier, less tapering. Its nucleus is in the middle of the body, while in Tr. Lewisi it is in the anterior half. The undulating membrane does not have folds in it, but appears more rigid than that of Tr. Lewisi.

Mode of infection. — The mode of infection is not known, but may be by some ectoparasite. In experimental work it is almost impossible to infect frogs. Laveran and Mesnil succeeded once out of seven or eight times, while up to that time it was thought impossible.

Trypanosomata Diemyctyli. — This parasite will be described in a special paper (see this number of the Journal).

REPTILIA. History. — From the existence of trypanosomata in the digestive tract of Ixodes testudinis, an ectoparasite of turtles, Leydig, 1857, concluded that they existed in the blood of turtles. Kunstler, in 1883, discovered a Trypanosoma in the blood of a mud turtle. In 1902 Laveran and Mesnil found in an Asiatic turtle a trypanosoma to which they gave the name of Tr. damoinæ.

Trypanosoma Damoinæ. —This organism is 32 μ long including flagellum, and about 4 wide. The protoplasm is finely granular with some larger chromatin granules toward the posterior end. Multiplication forms have not been seen.

Trypanosoma Lacertiliæ. — Gehrke has seen a trypanosoma in a lizard.

Trypanosoma Ophidiæ. — Dutton and Todd have seen trypanosomata in snakes and turtles in Gambia, Africa. They mention two varieties, one long, the other short.

Modes of infection. — The trypanosomata are spread by ectoparasites like Ixodes.

AVES. History. — Danilewsky published his article on Trypanosomata of birds in 1888. Laveran, in 1903, published his observations on Trypanosomata of the common brown owl (Syrnium ahico). Dutton and Todd described trypanosomata of Crithagra and Estrelda of Gambia, and Hanna wrote of the trypanosomata of the pigeon and crow of India. The trypanosomata Eberthi that Kent found in the digestive tube of the hen is a Trichomonas. Ed. and Et. Sergent, 1904, studied the blood of a large number of Algerian birds, and found them in only three species. Schaudinn, 1904, published his studies on alternation of generations and change of host of trypanosomata and Spirochætæ. In March, 1905, McNeal and Novy published the best work on trypanosomata of birds, and by means of pure cultures were able to prove that Schaudinn had "mixed cultures" when he evolved his ideas in the paper above mentioned.

Technic. — Blood for examination is obtained from the marginal vein on the inner side of the wing. The feathers are removed, the skin washed with a little water, and the small vein cut with a pair of sharp pointed scissors. The injury is so slight that the smallest bird can be examined every few days for some time. The detection of trypanosomata in fresh blood is difficult, as they are scarce, yet this method is, as a rule, more delicate than the examination of stained specimens because the motility of the organism is likely to draw attention to it, while in stained specimens the organism may be concealed by a mass of cells.

The bird trypanosomata do not stain readily. The nucleus may be colorless or nearly so. The flagella are usually very indistinct.

The injection of the trypanosoma cultures can be made subcutaneously or into the breast muscle. The intraperitoneal method is dangerous and was abandoned for intrapleural injections. For this purpose the needle of the syringe is inserted obliquely through the percular angle into the right pleural cavity. Relatively large doses, even one-half cubic centimeter, can be thus introduced into a small bird, such as a sparrow. The feathers over the wish-bone should be removed, and the skin washed previous to making injections.

Trypanosoma avium. — This form is found in blackbirds, bluebirds, blue-jays, orioles, robin, English sparrow, and song sparrow. There are two strikingly different forms, the multiplication, rosettes, and the free-swimming, darting, thread-like spirochætes. The spindle-shaped cells of the rosettes were 10 μ long and about 3 μ wide. The spirochæte form is 30 to 60 μ long and 5 to 1 μ wide.

Trypanosomata Mesnili. — The native trypanosoma is characterized by its large size and great bulk, and by a wide rounded posterior extremity. The length of the body is 50 μ, width, 8 μ.

Trypanosomata Laverani. — It is a wide spindle which measures 20 μ in length and 6 μ wide.

Tr. Johnstoni. — Dutton and Todd found this form in the estrelda estrelda. Its length is 36 to 38 μ, and 1 μ wide.

Tr. Paddæ. — In 1905 Dr. Levaditi found in the blood of a Padda aryzivora a new form. It is 30 μ long and 5 μ wide. The flagellum is very short.

Mode of infection. — This infection is spread by the *Culex pipiens* and may be fatal as seen in paddæ.

MAMMALIA. Trypanosomata Lewisi. — Mention is made as early as 1845 of trypanosomata in rats and hamsters. In 1850 Chaussat saw the organism in *Mus rattus.* The parasites were rare in young rats, but were nearly always present in the adults. In 1877 Lewis found the organism in the brown rats of Calcutta. The first good description of Tr. Lewisi is that of Crookshank in 1886. Then followed the work of Rabinowitsch and Kempner in 1899, Wasielewski and Senn, 1900, Laveral and Mesnil, 1900–1901, Jürgens, 1902, Francis, 1903, Martini, 1903, McNeal and Novy, 1903, McNeal, 1904, and Smedley, 1905.

Geographical distribution. — Tr. Lewisi has been found in every part of the world. Crookshank found twenty-five per cent of London rats infected. Rabinowitsch and Kempner found forty-one per cent infected in Berlin. Laveran and Mesnil found four per cent infected in Paris. At Krommenie, Holland, ninety per cent were infected. At Bordeaux, one hundred per cent; in India, twenty-nine per cent; in Manila, twenty-five to sixty-five per cent were infected. In Japan, South America, and Madagascar the infection is present. In this country, Ann Arbor, Philadelphia, Detroit, Lincoln, Nebraska, San Francisco, have infected rats, while none have been found in Washington and Boston.

Trypanosoma Lewisi. — The adult Tr. Lewisi is 27–28 μ long, flagellum included, and 1.5 to 2 μ wide. Unlike the dividing forms, the whole body takes part in the undulating movement. The posterior tip is often feebly stained. The centrosome is situated about 3 or 4 μ from the posterior tip. It measures 1 μ in length by 2/3 μ in width, and is placed transversely, extending across the entire width of the cell at this point. The flagellum at times appears to originate in this body, but the study of more favorable specimens clearly shows a chromatic space between them. There is an intimate connection, however, for these structures remain attached when all the rest of the cell has disintegrated. The flagellum extends in a smooth curve along the convex border of the parasite, supporting the undulating membrane, and is prolonged about 7 μ beyond the anterior tip as a free whip. The oval nucleus presents a chromatic network in a lighter nucleoplasm. At times a grouping of the chromatic into twelve chromasomes is apparent.

Smedley gives the following differences between the cultural and parasitic forms:

Cultural forms.	*Parasitic forms.*
Exceedingly active.	Very active.
Very variable in size and shape, generally spindle or pear-shaped.	Size only varies within narrow limits. Body is slightly fusiform, and has sharp pointed extremities.
Nucleus variable in position.	Nucleus is invariably situated at the middle of the anterior half of the body.
Centrosome is found close to the nucleus, or at a variable distance anterior to it. It is usually elongated.	Centrosome is found at a short distance from the posterior extremity, and is usually round.
Undulating membrane is not developed.	Undulating membrane is well developed, and is usually thrown into one or two folds.
Flagellum is frequently very long. Its basal portion is very short, owing to the position of the centrosome.	Flagellum much shorter, relatively to the length of the body of the parasite.

Susceptibility of other animals to Tr. Lewisi. — Tr. Lewisi, when injected into guinea-pigs, increases slightly, and then gradually disappears. The organism will live in no other animal.

Active immunity. — A single infection with Tr. Lewisi renders the rats free from parasites thereafter. Francis notes an exception, but the second infection lasted only three days.

Passive immunity.— It has been found that the serum of rats which have been immunized by one or more inoculations of trypanosome blood does give protection to other rats within certain limits. If one cubic centimeter of immune serum is added in vitro to one cubic centimeter of trypanosome blood, and the mixture injected into a fresh rat, no infection will follow.

Mode of infection. — The infection is spread by fleas and probably in the case of wild rats by the eating of infected rats, as rats are prone to eat each other.

Trypanosomiasis of smaller mammals. — Trypanosomata have been found in the mouse, the rabbit, the guinea-pig, the hamster, the mole, and other small mammals.

TRYPANOSOMIASIS IN THE HORSE. — There are five well-known forms of Trypanosomiasis in horses: Nagana, Surra, Mal-de-Caderas, Dourine, and Trypanosomiasis of horses in Gambia.

Nagana. — The duration of the disease produced by Tr. Brucei varies with the species of animal. From this point of view one can divide mammals into three groups. 1. Animals in which it produces an acute disease: mouse, rat, vole, marmotte, hedgehog, dog, monkey. 2. Animals in which it produces a subacute disease: rabbit, guinea-pig, wood-mouse, dormouse, equides, pig. 3. Animals in which it produces a chronic disease: cattle, goats, and sheep.

Geographical distribution. — Nagana is confined to Africa and the Island of Mauritius. In Africa it is found in German and English East Africa, Congo, Nubia, Somaliland, Soudan, Zambesi, and Zululand.

TRYPANOSOMA BRUCEI. — The differential characteristics of this organism are summarized by Smedley as follows:

CULTURAL FORMS.

Tr. Lewisi.	*Tr. Brucei.*
1. Spindle-shaped or pear-shaped. Very variable in size, usually 3 to 5 or 14 to 16 μ excluding the flagellum. Smaller and larger forms frequently seen.	1. Resemble forms found in blood of infected animals, but shorter and more pointed. More constant in shape and size than Tr. Lewisi, measure 15 to 20 μ excluding the flagellum.
2. Move with greater rapidity, generally in straight lines. The body is not curved or bent.	2. Movements much shorter and are generally of a wiggling character.
3. Protoplasm clear and homogeneous, rarely it contains a large single vacuole.	3. Protoplasm soon becomes slightly granular, invariably contains two or three large vacuoles.
4. Flagellum very long and active, often quite rapid except where it issues from the body.	4. Comparatively short but very active.
5. Undulating membrane absent, unless it is developed in a minute form at base of flagellum.	5. Well developed; its contractions passing around the cell in a spiral direction.
6. Colonies form large masses of cells which are symmetrically arranged with their anterior extremities directed centrally. Huge colonies visible macroscopically as small whitish granules are formed by the coalescence of several colonies.	6. Colonies of small size and much less numerous. The younger colonies may present a symmetrical rosette-like appearance, the flagella being directed outward. In older colonies the Tr. are closely packed together but somewhat irregularly arranged. Secondary massing together of the colonies does not occur to any extent.
7. Tr. multiply rapidly. Cultures swarm with colonies and free forms and remain alive for	7. Tr. are never so numerous and degenerate rapidly, the culture being generally dead at

three months or longer. Cultures retain their virulence for some time.	the end of two months. Cultures rapidly lose their virulence, the time taken to do so depending on the temperature of incubation.

STAINED PREPARATIONS.

8. Protoplasm stains pale blue.	8. Protoplasm stains a deeper blue and frequently contains deeply stained granules.
9. Centrosome usually rod-shaped and situated either at margin of nucleus or just anterior to it.	9. Centrosome much smaller, round or elongated, and often difficult to distinguish from the other granules. Generally situated at the posterior end.
10. Flagellum long and thick and projects for almost its entire length from the cell. It measures two to four times the length of the body.	10. Flagellum is short and fine and can be traced backward along the free border of undulating membrane to end in centrosome. It measures three to five microns.

Symptoms. — Bruce describes the symptoms of Nagana as follows: Fever of a remittent or intermittent type; catarrhal secretions from the nose and eyes; staring of the coat and edema of the abdominal region, the prepuce and the posterior extremities. The animal becomes markedly emaciated and has a dejected appearance, the head hangs, the hair becomes very rough, and in places falls out, the mucous membranes of the eyes and genitals become very pale, and there is generally a slight opacity of the cornea. Just before death the animal falls to the ground and dies apparently without suffering.

Pathology. — Bruce mentions the anatomic lesions in Nagana as a gelatinous atrophy of the subcutaneous tissues, subserous and subcutaneous ecchymosis and an enlargement of the spleen.

Mode of infection. — The disease is spread by the bite of the tsetse fly (Glossina morsitans).

Treatment. — Arsenic is used and also trypanred. Human serum, serum from animal origin, and serum from animals immune to Trypanosomiasis.

SURRA. — This form of Trypanosomiasis is scattered over the greater part of the world. It occurs in India, Annam, Korea, Persia, Java, Philippines, Abyssinia, Egypt, Madagascar, Togo, Chili, Marajo Island, Paraguay, Uruguay, in North America and Australia. It is caused by Tr. Evansi.

Tr. Evansi. — The surra organisms differ from the Nagana in the following respects: 1. They are larger, their average length is 25 to 35 μ, the flagellum is very long. 2. They are very actively motile and moved either backward or forward. 3. The protoplasm of the anterior portion of the cell contained a large number of small granules or globules about .3 to .5 in diameter, of a yellowish or greenish tint; in forms dividing longitudinally, these globules are arranged in parallel lines, one row in each half of the dividing parasites. 4. There was an entire absence of rosettes and colonies.

Symptoms. — The period of incubation is apparently six to eight days. The coat is staring, the submaxillary glands may swell, emaciation is constant, lacrymation may be profuse toward the end of the disease, petechiæ on the eyelids, hemorrhages into the anterior chamber, and opacity of the cornea are present, there are cartarrhal discharges from the nose, petechiæ, and injection of the nasal septum, ulcerations inside the nose, thickening of schneiderian membrane, stringhalt, muscular tremors and crossing of the legs are due to some effusion on the spinal cord or on particular nerves; there is often a voracious appetite and great thirst. Gurgling and tympanitis are present. The temperature is remittent, intermittent, and sometimes a relapsing type.

Pathology. — There are marked anemia and leucocytosis, serous effusions into the peritoneal cavity, petechiæ, jaundice, fatty changes in the muscles, liver, spleen, kidneys, and other organs, with a large deposit of fat under the skin.

Complications. — The complications are pneumonia, nephritis, pleurisy, sudden collapse, palpitation, pericarditis, hemorrhages, miscarriage, edema of the extremities and under surface of the abdomen, and effusion in the joints.

Mode of infection.—The disease is spread by flies—Tabanus tropicus and Stomoxys calcitrans.

DOURINE.—This disease occurs in Syria, Algeria, Morocco, Tripoli, Bohemia, France, Germany, Hungary, Spain, Turkey, and the United States. It is caused by Tr. equiperdum.

Tr. Equiperdum.—It is 25 to 28 μ long, being shorter and smaller than Tr. Brucei. The protoplasm is less intensely colored than the other pathogenic trypanosomata. The protoplasmic granulations are never observed as in the case with Tr. Brucei.

Symptoms.—This disease has a chronic and an acute form. The chronic form has three stages: 1. The stage of edema in the genital and abdominal regions. 2. The period of plaques, places that look as if a piece of metal had been slipped under the skin, as large at times as the palm of the hand. 3. The period of profound anemia and paraplegia.

Mode of infection.—It is usually spread by coition, rarely by biting flies.

Mal-de-Caderas.—This disease is limited to South America. The trypanosoma that causes it was discovered by Dr. Elmassian in May, 1901, Dr. Voges, Oct. 3, 1901, confirmed. It is caused by the Trypanosoma equinum.

Tr. Equinum.—In the fresh state Tr. equinum is very much like Tr. Evansi or Tr. Brucei. It is 22 to 24 μ long and 1.5 μ wide. Multiplication forms become 28 to 30 μ long and 5 μ wide. The characteristic feature is the centrosome, which is so small that its presence has been denied.

Symptoms.—Horses are never known to recover from this disease. It is a chronic disease lasting two to five months in the horse, and six to twelve months in asses and mules. There is an intermittent fever, rapid progressive emaciation, albumen and blood in the urine, the red blood corpuscles diminish and the microscopic changes of pernicious anemia appear; the lymphocytes and especially the eosinophiles increase in number; the most marked symptom is a symmetrical or asymmetrical paresis of the hind legs; defæcation and urination difficult; dyspnea; appetite and extreme thirst to the last; gradual extension of paralysis to other parts of the body and frequent edema.

Pathology. — Enlargement of spleen and lymphatic glands; serofibrinous exudations in body cavities; liver enlarged and engorged with blood; heart flabby; lungs often with hemorrhagichoids and subpleural emphysematous patches; catarrhal condition of the respiratory passages and pathologic changes in the spinal cord.

Treatment. — Treatment without success. Quinine, methylene blue, arsenic, enterol, potassic permanganate and other drugs have been used.

Mode of infection. — By biting insects in the rainy season when flies are most abundant. These varieties are Tabanus and Stomoxys calcitrans.

TRYPANOSOMIASIS OF HORSES OF GAMBIA. — This disease was discovered by Dutton and Todd in 1902. There were few horses affected, and they came from Senegal. It is caused by Tr. dimorphon.

Symptoms. — The symptoms differ in that there is no edema, and no staring of the coat. The parasites are very few in the blood and may be absent for long periods of time. At the end of the disease, the animal is extremely feeble, respiration very painful, sweating continually, with light convulsions just before death.

Tr. dimorphon. — There are three forms, a tadpole form, a long form, and a stumpy form. The tadpole form is 1 to 3 μ long, the long form 26 to 30 μ and the stumpy form 16 μ and 3 μ wide.

Mode of infection. — It is spread by the Glossina palpalis perhaps, but it is not known.

GALZEIKTE. — This disease occurs in cattle and is epidemic in South Africa, in Transvaal. It is produced by the Trypanosoma theileri, which is a distinct organism producing a distinct form of trypanosomiasis. All attempts to infect other animals have failed.

Tr. Theileri. — The large forms measure 60 to 70 μ long and 4 to 5 μ wide; the small forms are 25 to 30 μ long and 2 to 3 μ wide.

Symptoms. — There is an acute form of this disease and a subacute form. In from three to five days after inoculation there is a fever which lasts a number of days and then returns to normal. The symptoms are those of a severe anemia and the mortality is about twelve per cent.

Pathology. — The anemia and the enlargement of the spleen and lymphatic glands are the striking features of this disease. The heart is flabby with subserous petechiæ. The lungs present a hypostatic edema. The spleen is enlarged and soft.

Mode of infection. — The disease is spread by Hippobosca rufipes, which is very common in South Africa.

SLEEPING SICKNESS. — Sleeping sickness is a disease caused by Trypanosoma Gambiense. It occurs in Africa only. The first account of the disease was given by Winterbottom in 1803, who observed it on the west coast of Africa. From that time to the present it has been described by many observers. In 1902 Dutton and Todd published their work in Senegambia, which gives the first modern account of the organism and the disease.

Tr. Gambiense. — The organism is 17 to 28 μ long and 1 to 2 μ wide. The protoplasm often contains chromatic granules which are distinguished by their size and number. They are often present in pairs.

In every case of sleeping sickness the organisms are found in the blood or cerebro-spinal fluid or both. There is a very evident connection between cases with slight symptoms and those with symptoms of an advanced stage. The latent period may be as long as two to five years or longer. The change from the latent stage to the advanced condition is very gradual. The duration of the disease after its recognition by friends is from two to four months. No native who has shown definite and constant signs of ill-health has recovered.

Dutton and Todd say that they have only observed eight fatal cases of Congo sickness in which necropsies showed no obvious secondary infection. They do not think they have sufficient evidence to state that death is produced by trypanosomata alone. Secondary bacterial infections seem to determine the fatal issue of many cases.

Complications. — The complications are: Purulent meningitis, pleurisy and pneumonia, pneumonia and localized tubercle of lungs, localized gangrene of lung, enlarged caseating and breaking down glands in abdomen, dysenteric ulceration of bowel, universally adherent pericardium, and infiltration of pus in femoral, inguinal, and internal glands (gonorrheal).

Mode of infection. — It is spread by Glossina palpalis.

Koch divides trypanosomata into two groups: one composed of Tr. Lewisi and Tr. Theileri, the other of the remaining trypanosomata. These groups are distinguished by the fact that the organisms of the first are constant in their morphological characteristics, their virulence and their relation to domestic animals. Rat trypanosomata can be passed on exclusively to rats, and Theiler's organism to no other species of animal but cattle. Hence he draws the conclusion that they have been exclusively restricted to their own particular host, and consequently are most particularly suited to their host, thereby acquiring special properties and becoming a distinct species.

Quite different are the relations of the trypanosomata of the second group, to which Tr. Brucei, Tr. Evansi, Tr. equinum, and Trypanosomata of man belong; they are not sharply separated morphologically from each other, their virulence fluctuates over a wide range, and they are not exclusively found in one host. From these facts he draws the conclusion that the parasites of the second group have lived but a short time in their host, that they are not completely adapted to it, and have not yet developed into a distinct species.

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The classification used in this compilation is that of Weysse.

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TRYPANOSOMIASIS OF THE NEWT.*

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So far as I can learn, no mention has been made of trypanosomata in newts. Of closely related animals, the frog has often been found infected, while attempts to find the parasite in mud puppies (Necturus maculatus) have been in vain.

The newts about to be described were bought in an animal store in Boston, but I was unable to learn the place from which they originally came. These newts (Diemyctulus viridescens) had certain characteristics which distinguished them from healthy newts; they were of a darker color, the spots on the side appeared a brighter red and the shoulder and pelvic girdles were very prominent, owing to their lack of flesh. The abdomen was much swollen at times. They seemed as active as the healthy newt, and one specimen lived two years in the laboratory, although several of them died soon after they were purchased. They all had trypanosomata in large numbers at all times.

The trypanosomata were examined in the fresh state by drawing the blood with a hypodermic needle from the femoral artery of the newt. Blood could in this way be repeatedly taken without killing the animal. The drop taken was immediately diluted with physiological salt solution and a hanging drop made. Experiments with a two per cent magnesic sulphate solution proved it was good for preserving the blood corpuscles, but it killed the parasites.

In the fresh state the organism is very active and moves with a spiral motion. The whole body is contractile.

A number of different stains were tried, but the most satisfactory was Wolbach's modification of the Romanowsky stain. This is the same as Wright's modification except commercial wood alcohol is used instead of pure methyl

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alcohol. Specimens stained by this method show a blue protoplasm, a red flagellum and thickened edge of the undulating membrane, of which the reticulated structure is brought out clearly, a red nucleus, and a reddish posterior extremity with round unstained spaces in it. In the center of one of these spaces is the centrosome, which is of a dark color and is connected with the thickened edge of the undulating membrane. In addition to this connection between the centrosome and the edge of the undulating membrane, there may be one or two other lines connecting the centrosome with the edge of the clear space as shown in Fig. 2. The centrosome is small and often difficult to see. The nucleus is near the center, usually slightly posterior to it.

In stained specimens there are three different forms, the wide, the long, and the short. The first two seem but different phases of a contractile state. Fig. 4 shows the three forms drawn to the same scale with the camera lucida. The parasite is 45 to 50 μ long and 2 to 5 μ wide with a flagellum 24 μ long.

Inoculations into uninfected newts were always unsuccessful, as were inoculations into mud puppies. Several attempts were made to infect frogs, but in only one case was there an apparent infection. In this one an organism appeared with a flagellum in constant motion in front and one at the side. This corresponded to the dividing forms of McNeal and Novy. The infected frog died the next day.

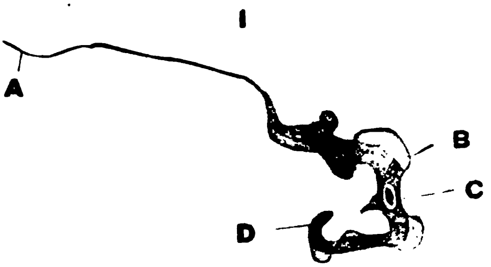
EXPLANATION OF PLATE XV.

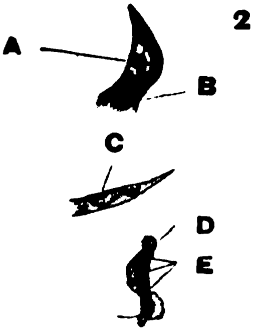
FIG. 1. A: Flagellum. B: Undulating membrane. C: Nucleus. D: Centrosome.

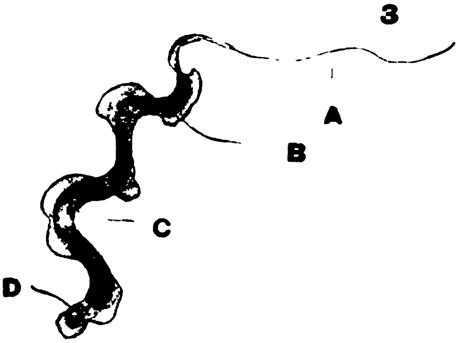
FIG. 2. Shows the vacuolated space at the posterior extremity with the thickened edge of the undulating membrane crossing this space and joining the centrosome. There are two other lines crossing the vacuolated space. No connection between the centrosome through the vacuolated space to the thickened edge of the undulating membrane has been shown before, though the centrosome with the thickened edge attached has been found in rat's blood (free from the body of the trypanosome). A: Centrosome. B: Thickened edge of the undulating membrane. C: Centrosome. D: Centrosome. E: Vacuoles.

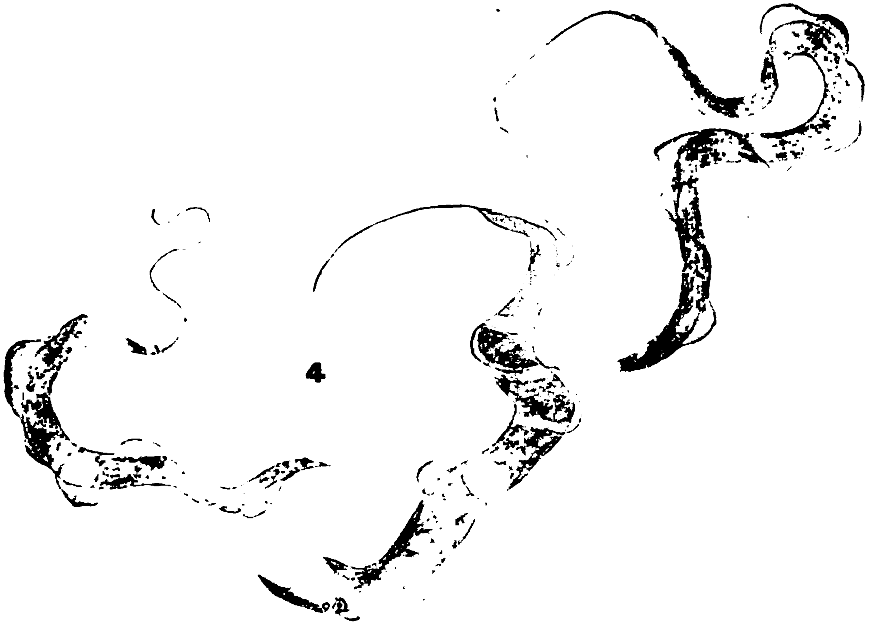
FIG. 3. A: Flagellum. B: Undulating membrane. C: Nucleus. D: Centrosome.

FIG. 4. Drawn to same scale to show the differences in size.









TOBEY.

A NEW TRYPANOSOME.

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THE RELATION OF AUTOLYSIS TO THE HISTOLOGICAL CHANGES OCCURRING IN NECROTIC AREAS.*

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In 1900 Jacoby[1] found that if a portion of a dog's liver was ligated off and the dog kept alive for some time afterward, the necrotic isolated portion contained free leucin and tyrosin. This was the first actual demonstration that the retrogressive changes in areas of anemic necrosis are associated with, and presumably the result of, enzymotic autolytic processes. Since that time it has been generally accepted that the softening and absorption of necrotic tissues is the result of autolysis by the proteolytic enzymes contained within the degenerated cells, and numerous chemical investigations have shown that in gangrenous tissues, suppurating areas, resolving exudates, degenerating tumors, and other similar conditions the products of proteid digestion may be found. There can be little question, therefore, that autolysis is an important factor in all processes associated with the removal of areas of degeneration, but as yet there has been little consideration of the relation of autolysis to the histological changes that occur in such areas, nor has it been determined to what extent the autolysis is accomplished by (1) the intracellular enzymes of the necrosed cells; (2) by the enzymes of invading leucocytes, and (3) by the constituents of the blood plasma. Neither do we know to what extent the action of these digestive agents is modified by various conditions and substances existing within the body.

A few studies of the histological changes occurring in aseptically and antiseptically preserved pieces of organs kept for varying periods outside of the body have been made, but most of them antedate the general appreciation of the intracellular proteolytic enzymes. Meissner[2] observed many years ago that aseptically preserved organs retained their general

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structural characteristics for many weeks, but the finer structure seems not to have been considered. Hauser[3] studied the structural changes more closely, and noted the loss of nuclear staining, while the general cell form remained relatively well perserved; he observed particularly the appearance of fat in the cells, and of fat crystals on the surface of the tissues, and considered that he had found evidence of "fatty degeneration" of proteids. Consequently, because of the warm controversy over the problems of fatty metamorphosis, the observations on the histological changes in autolyzing tissues have been directed particularly towards the fatty substances that may become visible and stainable, rather than towards the fundamental questions of cell necrosis. The first systematic study of the histological changes in autolysis was that of F. Kraus,[4] who considered particularly the question of fatty changes and came to the conclusion that no actual increase of fat occurred during autolysis (it may be stated here, parenthetically, that the general result of the more recent discussion of this phase of the question has been in agreement with Kraus' results; *i.e.*, during cellular autolysis fat that is normally invisible may become visible and stainable, but there is no actual increase in the fat in the tissues, and therefore no "fatty degeneration" of proteids).[5] He also noted as an early and characteristic feature of autolysis the disappearance of the nucleus, which occurred in the large parenchyma cells of the glandular organs, and stated that this change is identical with the change described in necrosis by Cohnheim and Pfitzner (the karyolysis of Klebs), and therefore is solely a post-mortem alteration. This was in direct contradiction to the view of Weigert that the "Kernschwund" was characteristic of coagulation necrosis, and required a vital suffusion of the tissues with blood plasma, and aroused much opposition.[6] A new phase was then added to the interest in the changes occurring in dead tissue, and as a result a number of investigators compared the histological alterations observed in autolyzing tissues with those described by Weigert, Ribbert, Israel, and others in experimentally produced areas of anemic necrosis. This was particularly done by Schmaus and Albrecht,[7, 8] who found that the changes in autolysis are in the main quite the same as those observed in infarcts, including not only the usual nuclear solution (karyolysis), but also the karyorrhexis, which had been considered as a more vital process, although this last is not so prominent in vitro as it often is in anemic necrosis.

All of these observations were made before the work of Salkowski, Jacoby, and others had led to a general understanding of the essential features of cellular autolysis, and the changes observed were referred to, as a rule, simply as "postmortem change," and ascribed generally to a dissolving of the cell components by the fluids of the plasma. More recently we find the histological changes of autolysis studied more definitely, as a result of the action of the intracellular enzymes, in the Tübingen laboratories. Dietrich and Hegler[9] examined various organs permitted to undergo autolysis, either preserved in a moist condition or in physiological salt solution, considering particularly, however, the relation to fatty changes and the appearance of "myelin" forms. They studied liver, kidney, heart muscle, and psoas muscle, and found in general much the same alterations that were observed earlier by Kraus, Goldmann, and others. The liver shows more rapid and extensive changes than the kidney, which preserves its general gross structure fifty-six days or more, while the heart muscle undergoes changes much more slowly. There seemed to be no essential difference whether autolysis occurred in salt solution or in a moist chamber. Dietrich seems inclined to question the importance of autolysis in causing the nuclear changes, referring to autolysis as a "Schlagwort." Pycnosis he ascribes to loss of water from the nucleus to the cytoplasm; if the nuclear substance is soluble in the cytoplasm karyolysis occurs, if insoluble we get karyorrhexis. He does not, however, explain the cause of the changes in the intracellular physico-chemical conditions that follow cell death. Richter[10] found that pieces of liver, kidney, and muscle placed in the abdominal cavity of an animal (either free or in collodion sacs) undergo changes similar to those observed in similar tissues preserved aseptically in the incubator, but was particularly interested in the peripheral deposition of fat observed in these tissues.

We have, then, evidence that the changes occurring in infarcts and in other necrotic areas are similar to those that occur during autolysis in vitro, but there remain a number of interesting problems to solve. For example, if we examine an aseptic anemic infarct of the kidney of some standing we find that the removal of the dead tissues goes on very slowly, and apparently solely from the periphery. Thus in a renal infarct, the age of which could be definitely stated to be fourteen weeks, there remained still a layer of necrotic cortex about one millimeter thick that had not been absorbed, and under the microscope it could be seen that the epithelial lining of the unabsorbed dead tubules was quite as thick as that of a recent infarct. On the other hand, in a kidney undergoing autolysis outside the body the epithelium of the convoluted tubules will be found in twenty days to have lost over three-fourths of its volume, and in sixty days it will have almost entirely disappeared. Evidently, then, cells do not become digested as rapidly by their own enzymes in infarcted areas as they do in vitro; indeed, there is little evidence that in small infarcts the intracellular enzymes digest the necrotic cells at all. On the other hand it can be readily seen that the absorption of the infarcted area occurs from the periphery, and is associated with a slow invasion of leucocytes and proliferating cells. Apparently the changes that occur in an aseptic necrotic area do not lead to the production of chemotactic substances in any considerable amount, for the failure of leucocytes to invade aseptic necrotic areas is rather striking. Indeed, there is much less infiltration of such necrotic tissue by leucocytes than is observed in the case of chemically inert foreign bodies (*e.g.*, Adler's experiments with elder pith[11]), which would suggest that possibly negatively chemotactic substances may be present in the infarcts. In support of this may be mentioned the demonstration by Magnus-Levy[12] of lactic acid among other substances produced in autolysis, and lactic acid has been shown repeatedly to be one of the most active substances exerting "negative chemotaxis" in the usual sense of the term.* In any event, it would seem that the absorption of aseptic dead tissues does not depend upon autolysis by the intracellular enzymes so much as has been generally assumed, but is usually the result of heterolysis by invading leucocytes. Hence aseptic areas undergo softening and solution extremely slowly unless something happens that causes an invasion of leucocytes. The failure of absorption characteristic of tuberculous areas may depend upon a destruction of the autolytic enzymes of the dead tissues (a matter that has yet to be investigated) or, more probably, upon the absence of positive chemotaxis and the consequent failure of leucocytic invasion. Certainly it is not due to the conversion of the proteids of the dead cells into an indigestible modification, as has been suggested, for whenever leucocytic invasion of caseous material is brought about through infection (or by injection of iodoform emulsion), the softening of the necrotic tissue goes on very rapidly.

In considering the manner in which infarcts are absorbed, as outlined above, a number of recently described features of autolysis were found to apply to the conditions observed in the infarcts. First of all may be mentioned the observations of Hedin, Wiener,[13] and Schryver,[14] that a certain degree of acidity is favorable to autolysis, which process goes on relatively very slowly if the reaction is kept neutral, and is completely checked by so slight a degree of alkalinity as that of a 0.2 – 0.4 per cent solution of NaOH. When tissues undergo autolysis in vitro considerable amounts of organic acids are produced (Magnus-Levy), but in the body these acids presumably diffuse out and also are neutralized by the alkalies of the plasma, which are perhaps sufficient to impede if not to check autolysis. In support of this hypothesis may be mentioned that, in large areas of aseptic anemic necrosis, softening occurs much more rapidly and chiefly in the center, which may, perhaps, be due to an accumulation of the organic acids in the central part rendered possible by the distance from the alkaline plasma of the circulating blood. Secondly, we have the observation that normally the blood plasma contains anti-enzymes, not only for the enzymes of the digestive secretions, but also for the intracellular autolytic enzymes. These have been studied particularly in relation to the autolysis of exudates by Opie,[15] who found that the serum of inflammatory exudates has a marked inhibiting effect upon the autolytic action of the leucocytic enzymes. Furthermore, he found that the leucocytic enzymes differ from those of the fixed tissue cells (excepting bone marrow, which resembles the leucocytes)[16] in being most active in an alkaline medium, although the action of the anti-enzymes is destroyed by acids. Schryver[17] has found that the serum also retards autolysis of liver tissue. It would seem quite probable that these anti-enzymes may be the cause of the failure of autolysis of necrotic areas, and also of their slow peripheral digestion by the leucocytes, except in the case of an excessive accumulation of leucocytes out of proportion to the amount of inhibiting plasma present at the same time. The more rapid digestion of the central parts of large necrotic areas might be referred to this lack of inhibiting substance, as well as to the development of an acid reaction — both factors working conjointly in favor of central and against peripheral autolysis.

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* It has been shown by Hektoen (Jour. Amer. Med. Assoc., 1906 (xlvi) 1407) that substances of this nature appear to cause their chemotaxis-inhibiting effect by producing an alteration in the opsonins. It would be interesting to ascertain the effect of the products of autolysis upon the opsonins.

In order to test the relation of the inhibiting action of the serum upon autolysis, studies were made of tissues allowed to stand for varying lengths of time in serum that had been heated, and compared with tissues from the same animal placed in fresh serum (serum and tissues were obtained from the same animal to render conditions as nearly natural as possible, the bleeding of the animal at the same time reducing the amount of residual blood in the organ to be examined). As most investigations of this kind have been made with tissues kept in physiological salt solution, organs kept in this medium were also studied, and, as will be shown later, gave quite different results from those obtained with the tissues kept in serum. This might well be expected in view of J. Loeb's demonstration of the profound modification of physiological processes by an excess of one variety of ion, and is but one of a number of evidences that in many experimental studies the assumption that physiological salt solution is an inert substance, not modifying the results of the experiment, is unwarranted. As the histological changes of autolysis are most striking in the kidney, because of its variety of functionating cells, the chief results of an experiment with this organ are given below.

A large dog was made unconscious with morphine, and bled to death. The blood was whipped free from fibrin, and the serum separated by centrifugalization. One-half was heated for thirty minutes at 80° C. and separated from the resulting coagulum by filtration and expression. Pieces of tissue of suitable size for sectioning (about 1.5 centimeters square and one centimeter thick) were taken from the same dog, and placed in: (A) sixty cubic centimeters of heated serum plus five cubic centimeters of toluol; (B) sixty cubic centimeters of unheated serum plus five cubic centimeters of toluol; (C) sixty cubic centimeters of 0.85 per cent NaCl solution plus five cubic centimeters of toluol.* These stood in stoppered bottles at 20° C. for varying periods of time, and were hardened for examination in Zenker's fluid. Staining was by hematoxylin and eosin. The results were as follows:

Eight hours. Fresh serum. — Epithelium of the convoluted tubules slightly swollen, nuclei stain a trifle deeper and more diffusely. All other structures appear normal, including the red corpuscles.

Heated serum. — Epithelium of the convoluted tubules at the surface of the specimen seems cleft, nuclei often distinctly pycnotic or in some cases pale or quite unstained; deeper in the tissue the changes are similar but less marked. Other tissues unchanged.

Salt solution. — Appears quite different, in that near the periphery the epithelium seems to have been dissolved out to a great extent, leaving a granular material filling the lumen of the tubules. Where protected by fat tissue in the capsule this change has not occurred, showing that it is due to a solvent action of the salt solution. Deeper in there is less change, but some of the epithelium of the convoluted tubules stains deeply with eosin, resembling coagulation necrosis, or sometimes stain diffusely pale purple, as if containing dissolved nuclear material.

Twenty hours. Fresh serum. — Epithelium of convoluted tubules generally much swollen; cytoplasm coarsely granular and stains well with eosin; most of the nuclei have disappeared, those remaining being for the most part small and staining deeply and diffusely; in a few tubules a diffuse, faint, blue-staining substance in the lumen. The epithelium of the collecting tubules and the limbs of Henle's loop show some pycnosis. All other structures are practically unchanged.

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*There is no evidence that toluol causes appreciable modifications in the tissues.

Heated serum. — Changes are all much more marked than in the fresh serum specimens. Convoluted tubules have lost all of their nuclei, the cytoplasm is granular and cleft, and there is a diffuse blue stain in the contents of the lumen. Collecting tubules show much pycnosis, but in the medullary rays there is often found a diffuse blue staining as if the nuclear substance had diffused through the cytoplasm; the same changes are observed in the limbs of Henle's loops. Glomerular tufts in many instances show some loss of chromatin substance and in the renal capsule the connective tissue nuclei are faint or absent, but the endothelium remains well stained. All staining is faint immediately about the periphery, but there is no such dissolving out of cell substance as in the salt solution specimens.

Salt solution — The dissolving-out process has extended deeply into the specimen, but affects only the larger forms of epithelial cells; apparently just before undergoing solution the epithelium swells and stains deeply with eosin, and then the central portion is dissolved, leaving a faintly stained granular débris in the lumen, while a thin margin of deeply stained cytoplasm remains near the basement membrane.

Forty-eight hours. Fresh serum. — Condition very similar to that of the specimen in heated serum twenty hours, except that there is no disintegration in the cytoplasm. Because of the preponderance of the changes in the convoluted tubules the specimen resembles the so-called "differential necrosis" observed in acute intoxications.

Heated serum. — Only a few nuclei remain in the cortical structures, the limbs of Henle's loops and the collecting tubules retaining about half of their nuclei in a pycnotic condition, while each glomerular tuft has but three to six nuclei remaining; even the nuclei of the stroma and the capsule are gone. In the pyramids the epithelial nuclei are very small and dark-staining, as also are those of the stroma. The nuclei of the vessel walls and endothelium still stain well.

Salt solution. — Differs from the heated serum specimen chiefly in the extensive dissolving out of cell substance; the nuclear changes are quite similar in most respects.

Seventy-two hours. Fresh serum. — Changes stand between those seen in the specimens kept in heated serum twenty-four and forty-eight hours. In the cortex the nuclei of the stroma and the smaller tubules still remain, although pycnotic, and the glomerular nuclei are nearly all present.

Heated serum. — In the cortex an occasional nucleus can be found in the limbs of Henle's loops. In the pyramids a part of the nuclei remain in a pycnotic condition, and often fragmented. About the periphery there is a zone from which all stain has been lost.

Salt solution. — Differs from the above chiefly in the greater loss of cell substance.

Ninety-six hours. Fresh serum. — Less disintegration than in heated serum specimen of forty-eight hours, about half of the nuclei still remaining in the glomerules and in Henle's tubules, and the nuclei of the basement membrane still stain well.

Heated serum. — Only a few nuclei left in the collecting tubules, and in the endothelium and muscular tissue of the larger vessels.

Salt solution. — Except for the excessive solution of the cytoplasm resembles the heated serum specimen.

Later changes. — After six days the fresh serum specimen is somewhat better preserved than the specimen kept in heated serum for but seventy-two hours; whereas the heated serum specimen of six days shows only a few pycnotic and fragmenting nuclei in the collecting tubules in the centers of the pyramids. After eleven days there are still some nuclei in the cortex of the fresh serum specimen, both in the stroma and in the smaller tubules. After twenty days not a nucleus can be found in the specimens kept in either heated serum or in salt solution, and the epithelium of the convoluted tubules has suffered a great reduction in volume by a dissolving out from the center. In the fresh serum specimen there is still little loss of cell substance, and an occasional pycnotic nucleus can be seen in the pyramidal epithelium.

We see, then, that there is a striking difference in the rate of autolysis in the specimens kept in heated and in those kept in unheated serum, which, in our present state of knowledge, can be best ascribed to the presence in the latter of thermolabile inhibiting substances, *i.e.*, anti-enzymes. The effect of the anti-enzymes is merely retarding, the changes in structure produced by the autolysis pursuing the same course, but requiring about double the time to reach the same stage (this time ratio of two to one seems to be quite constant so long as the nuclear changes can be used as an index). The tissues preserved in physiological salt solution show that this fluid has a marked solvent effect upon cellular constituents, particularly the cytoplasm, the central portion of the epithelium of the convoluted tubules being dissolved out early, especially near the surface of the specimens; otherwise the process of autolysis, so far as the nuclear changes are concerned, proceeds at about the same rate as in the specimens kept in heated serum. In the specimens kept in fresh serum the changes are much more similar to those observed in infarcts than in the specimens kept in either heated serum or salt solution, in that the nuclear changes far outweigh the cytoplasmic changes, which consist chiefly of a coarse granular alteration (recalling the "tropfige Entmischumg" of Schmaus and Albrecht), with little evidence of digestion or solution of the proteid constituents until after a period of three weeks, and then relatively very slowly.

The nuclear changes are various — they occur first in the large epithelial cells of the convoluted tubules, and last in the stroma, vessels, glomerules, and the epithelium of the limbs of Henle's loops. In the convoluted tubules little can be observed but a simple solution of the staining elements, leaving the structure of the nucleus still visible in an unstained condition. Occasionally in these cells there occurs a preliminary decrease in size of the nucleus, associated with a diffuse and greatly increased affinity for basic dyes, *i.e.*, pycnosis. The pycnosis is, however, much more marked in the nuclei that persist longest, especially in the epithelium of the collecting tubules, and occasionally in these tubules a considerable degree of karyorrhexis may be observed in the later stages. These changes differ but little from those described by Weigert, Ribbert, and others as occurring in experimental infarcts, especially if we compare only the specimens kept in fresh serum, which, of course, approach most closely the conditions in infarcted tissues. Sometimes the dissolved nuclear material seems to diffuse slightly before it has entirely lost its staining power, so that a diffuse basic stain may be seen in the lumen of the tubules, especially in the ascending limb of Henle's loop. This is not marked, however, and is almost entirely absent in the tissues preserved in fresh serum.

In comparison with the above statements we find that the changes occurring in the other organs and tissues of the body differ from those observed in the kidney only in rate of occurrence, and not at all in the nature of the histological changes. These may be summarized as follows:

Of the other organs the *liver* is by far the most rapidly affected, marked changes occurring in eight hours (in salt solution at 20° C.) and after thirty-four hours no nuclei whatever are left except in the walls of the larger vessels. The changes of cytoplasmic disintegration are also most marked in the liver both macroscopically and microscopically, the cells becoming greatly reduced in size and number, and after thirty days the single lobules are reduced to a very small size. Next in order of rapidity of change comes the *spleen*, which shows some pycnosis of the nuclei, and in twenty hours a diffuse tingeing of all the tissues by nuclear stain has occurred, associated with loss of stain in the pulp and in the periphery of the Malpighian bodies. In thirty-four hours all nuclei have lost their stain except those of the reticulum, trabeculæ, and vessels, and a few in the centers of the Malpighian bodies. In the *pancreas* the changes occur in certain foci, as if the trypsinogen had become activated in some areas before in others, and the resulting changes are quite different from those of simple autolysis in that all structures except the stroma are destroyed and severely disintegrated wherever the changes occur at all. After thirty-four hours practically all nuclei have disappeared, while the stroma exhibits the peculiarity often observed in autopsy specimens, that it stains strongly with the basic stain.* The first change observed in the *thyroid* kept in salt solution is an apparent partial solution of the colloid, associated with the formation of coarse granular flocculi, which persist for a long time. Nuclear changes in the epithelium appear first after forty-eight hours, and consist chiefly of pycnosis and irregularity of form; the nuclei do not disappear until from the fourth to the sixth day; the stroma nuclei last from ten to eighteen days, and the volume and density of the dead epithelium become reduced very slowly. The *lung* changes more rapidly than the thyroid, possibly because of the more superficial character of its structure in relation to the fluid in which it is preserved. The nuclei of the alveolar epithelium disappear in about thirty-six hours, as also do those of the bronchial mucosa. The nuclei of the cartilage and the vessels remain from six to ten days. *Muscle* tissue undergoes changes very slowly, especially in its cytoplasmic structures. In the *myocardium* the nuclei fade from the fourth to the tenth day, but the striations are still distinct after thirty days; striated muscle undergoes changes still more slowly. In some respects the *brain* is the most slowly changed of all the organs, for even after thirty days the nuclei of the cortical cells are still distinct, although not well stained. The cytoplasm of the ganglion cells seems to suffer much loss after two to three days, but their nuclei stain well up to eighteen days, at which time a large proportion of the nuclei of the white matter have disappeared. *Squamous epithelium* of the skin and hair follicles persists from ten to eighteen days, while the nuclei of the subcutaneous and fat tissues disappear in from six to ten days in the same specimens.

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* *Intestine* undergoes a rapid digestion commencing with the lumen and working outward, therefore due apparently to the action of its contents. In one specimen examined an intestinal worm was present which showed severe nuclear and cellular alterations within eight hours, when the lining epithelium of the intestine was still intact. This is interesting in connection with the observations of Weinland and others concerning the presence of antibodies for trypsin in the intestinal parasites.

By far the most resistant cells of the body seem to be those of the endothelium, and similar cells found about the walls of the larger vessels, probably the endothelium of the perivascular lymph channels; these persist in some tissues in a well-stained condition for thirty days or more. Apparently these and the other elements of the stroma and vascular system are not so much affected by their own enzymes, but are attacked by the constituents of the parenchymatous cells in their vicinity, for they disappear in the various organs in proportion to the rate at which the chief cells of these organs are themselves affected; thus in the liver none survive in a stainable condition for forty-eight hours, while in the subcutaneous tissue they last for a month or more. Schryver[18] has studied by chemical means the rate of autolysis in different organs and found that the liver and spleen digest themselves with about equal rapidity and nearly twice as fast as the kidney; next in order coming the myocardium, and then the voluntary muscle. These results are quite in accord with the histological changes as observed above.

According to the histological changes previously alluded to, the solution of the tissue elements in the necrotic areas in the living body seems to be accomplished largely by the leucocytes and little, if any, by the intracellular enzymes of the dead tissue cells; and conversely, the nuclear changes characteristic of necrosis seem to depend largely upon the intracellular enzymes and little upon the leucocytes. For the purpose of establishing this experimentally a number of experiments were performed for me by Mr. J. E. Tyree, to whom I wish here to express my indebtedness, which corroborated the results obtained by me in connection with another investigation. These consisted in implanting into the abdominal cavities of rabbits pieces of spleens obtained from other rabbits, or from dogs and sheep; in half of the experiments the tissues had been heated quickly to 100° C. in an Arnold sterilizer to destroy the autolytic enzymes, while in the other half the tissues were implanted in a fresh, aseptic condition. After varying lengths of time the tissues were removed and studied histologically, the results being briefly as follows:

The heated tissues undergo no changes whatever for a surprisingly great length of time. At the end of three weeks not only are all the nuclear and tissue structures absolutely unchanged, but even the red corpuscles appear well preserved. About the periphery is a narrow zone that stains faintly, this change affecting all elements alike. There seems to be absolutely no invasion by leucocytes, although a delicate capsule of connective tissue has been formed about the periphery. By the end of six weeks this pale zone has widened to a depth equal to from two to four times the thickness of the capsule of the spleen. In this area the nuclear stain is absent at the periphery, gradually appearing by gentle transitions as the deeper tissues are approached which stain with normal intensity. In the middle of the pale zone may be seen numerous hematoxylin-staining granules that are apparently the result of karyorrhexis; pycnosis is not observed. Between the encapsulating connective tissue and the spleen substance is a narrow zone where the latter seems to have been partly digested out by leucocytes that are present here in small numbers. In the unstained zone can be found also an occasional leucocyte, although so few that one questions whether they are responsible for the loss of staining. Yet, on the other hand, in a heated specimen that became infected, and as a result infiltrated with leucocytes, the nuclear stain had entirely disappeared in less than ten days (and *in vitro* experiments have shown that infection, at least with ordinary putrefactive organisms, does not greatly affect nuclear staining). Apparently, then, the leucocytes are able to cause solution of the chromatic substance of the heated nuclei, which does not seem to be accomplished by infiltrating plasma alone. The eventual solution of such heated tissues is accomplished as in aseptic infarcts, by leucocytes acting upon the peripheral portions, but not entering into and acting upon the deeper tissues.

In striking contrast to the heated specimens, the *fresh tissues* undergo rapid changes, just as they do *in vitro*, so that in the course of a few days the nuclear staining is lost. At the end of ten days the nuclei have all disappeared except those in the capsule, which remain almost unchanged at a time when the nuclei of the trabeculæ deeper in the organ have disappeared. This may be due either to an inhibiting effect of the plasma upon the autolysis, or to the presence of a sufficient amount of nourishment to sustain their vitality. The spleen pulp shows a diffuse basic staining, most marked at the site of the Malpighian bodies, which is apparently due to diffusion of nuclear substance. Granular brownish masses, apparently blood pigment, are also present, although shadows of blood corpuscles also persist; by the seventh week the pigment is more crystalline, peripheral leucocytic invasion by leucocytes has begun, and all traces of nuclear stain are lost from the spleen elements.

From these experiments we learn that nuclei of cells that have been killed by heat may remain for extremely long periods of time in the animal body without undergoing any appreciable changes, after nine weeks such tissues show only a slight peripheral loss of stain, the central nuclei remaining unaltered. At least two interpretations may be made of this fact: first, that the changes in the nuclei observed in areas of necrosis are brought about by the intracellular enzymes, which are destroyed by heating; second, that the heating so alters the nucleoproteids that they are not attacked by the agents that make them unstainable under ordinary conditions. Against the latter interpretation, however, may be advanced the following observations: (1.) As a rule, proteids that have been heated are more readily digested than are fresh proteids, either because of a change in molecular configuration, or because of the destruction of an antibody, or perhaps both. (2.) If the heated tissues become infected so that an invasion of leucocytes takes place the nuclear stain disappears promptly, showing that the nucleoproteids are still in a condition to be attacked by the leucocytic enzymes. It therefore seems that the most probable explanation of the results is that the disappearance of the nuclear stain that occurs in dead tissues depends upon the intracellular enzymes of the necrosed cells.

SUMMARY.

Putting together the results of the above experiments, we find evidence that in typical areas of anemic necrosis, such as infarcts, etc., the changes that occur are as follows:

1. There is first a decomposition of the nucleoproteids of the nuclei, which is probably brought about by the intracellular enzymes of the starved cells. Ordinary autolytic enzymes resemble trypsin, at least in the major effects produced, and trypsin has the power of decomposing nucleoproteids only to the extent of splitting off the proteid groups, leaving the nucleic acid in a free state (Sachs). If the autolytic proteolytic enzymes of the cells behave in the same way we may find here an explanation of the process of pycnosis, as follows: The density with which a nucleus stains seems to depend upon the extent to which the nucleic acid is saturated with proteids. Unsaturated nucleic acid is

strongly acid, and therefore has a marked affinity for basic dyes; the more the nucleic acid is saturated with proteids the less will be its affinity for basic stains.[19] Hence a decomposition of the nucleoproteids of the nuclei should, theoretically, cause an increased intensity of basic stain, which would be more diffuse than normally, as is the case in pycnotic nuclei. The cause of the decrease in size is probably the loss of nuclear substance through this splitting off of proteids. (Dietrich's attempt to explain pycnosis as due to an exosmosis of water from the nucleus into the cytoplasm may be well questioned on the ground that cell membranes seem to lose their semipermeable character immediately after the death of the cell.) The nucleic acid is subsequently so altered that it does not stain any more, which may be due either to its neutralization by the alkalies of the plasma, or to a further splitting, which last seems probable in view of Schmoll's [20] observation that in caseous material there is surprisingly little phosphorus, indicating that the nucleoproteids become so decomposed that the phosphorus is liberated in a readily diffusible form, which soon escapes from the necrotic area. Jones[21] has shown the existence of enzymes in the thymus and adrenal that have the power of decomposing nucleic acids into their constituents, phosphoric acid and the xanthin bases. Sachs[22] found a similar enzyme in the pancreas and called it "nuclease."* The view supported by numerous earlier histological investigators, that the chromatic nuclear substance is simply dissolved out by the blood plasma, is probably incorrect; it must first be decomposed into its soluble constituents. Apparently the leucocytes contain similar nucleic-acid-splitting enzymes, at least they seem to destroy chromatin substance in both fresh and heated substances very actively.

2. In infarcts the intracellular proteolytic enzymes seem to attack the proteid structure of the cell but little and very slowly, except in the center of large necrosed areas. It would seem that this depends chiefly upon the presence in

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* The subsequent changes that occur in the xanthin bases have also been further studied by Jones with Partridge and Winternitz, and by Schittenhelm.[23]

the blood plasma of antibodies or of some heat-susceptible substances which check the action of the autolytic enzymes. Possibly the alkalinity of the plasma also exerts a retarding influence.

3. Chemotactic substances do not seem to be formed in aseptic dead tissues; indeed the absence of leucocytic infiltration is so marked that it seems possible that substances with a negative chemotactic effect are present, such as lactic acid, which is known to be formed during autolysis (Magnus-Levy).

4. The slow absorption of infarcts, etc., seems to be accomplished almost entirely through a digestive action of the leucocytes acting slowly from the periphery; *i.e.*, the digestion is not so much through autolysis as through heterolysis (to use the term proposed by Jacoby[24] for the digestion of one sort of cell by the enzymes derived from another sort). The leucocytic proteases act best in an alkaline medium (Opie), which possibly explains their ability to attack substances impregnated with alkaline blood plasma. Leucocytes seem also to contain enzymes decomposing nucleoproteids, as shown by their effect on heated tissues in the experiments cited.

5. The rapidity with which autolytic changes occur in different organs and tissues, as indicated by the disappearance of nuclear staining, seems to be about as follows: (1) Liver, kidney (epithelium of convoluted tubules); (2) spleen, pancreas; (3) kidney (collecting tubules, straight tubules, glomerules); (4) lung (alveolar and bronchial epithelium); (5) thyroid; (6) myocardium; (7) voluntary muscle; (8) skin (epithelium); (9) brain (cortical cells). Stroma cells seem to be attacked chiefly by enzymes (or products) from the parenchyma cells, since they disappear from various organs in direct proportion to the rapidity of autolysis in the parenchymatous elements. Of all cellular elements the endothelium of the vessels seems to undergo the least autolysis and to be least subject to heterolysis by enzymes from other cells.

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A CASE OF BLASTOMYCOSIS: THE RESULTS OF CULTURE AND INOCULATION EXPERIMENTS.

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During the last twelve years, since attention has been directed to the subject of blastomycosis, a careful watch has been kept for examples of disease that might be classed in this category, in Boston. On account of the resemblance of tuberculosis verrucosa cutis to this affection, all cases which before would have been, without hesitation, classed as the latter affection have been examined with a view to blastomycosis. Hitherto, however, no instance had presented itself. The following case, which was observed during the last autumn, will be admitted to present a clinical picture quite in accord with the usual form of blastomycosis as described by Gilchrist, Hyde and Montgomery, etc.

Frank Pasquale, Boston. Married. Age 32 years. Born, Italy. Occupation, works with the pick and shovel. As patient cannot speak much English, the history is defective. He came to this country two years ago from Italy, where he had been employed on a farm. Entered the skin ward of the Massachusetts General Hospital, Oct. 4, 1905. Family history negative. Past history, small-pox some years ago; otherwise always well up to nine months ago. About nine months ago patient noticed "boil," so-called, on back of left wrist, which has slowly but progressively grown to assume present size and appearance. Lesion has never caused pain or itching. Appetite good. Bowels regular. Feels well. Sleeps well.

Physical examination. Heart, apex nipple line. Systolic murmur at apex and base, not transmitted to axilla or back. Lungs negative. Abdomen negative. Urine, normal, acid, 1028, no albumin or sugar. Over inner half of extensor surface of left forearm, just above the internal condyle, is an oval patch about two and one-half by one and one-half inches, surrounded by a dull red, smooth, slightly elevated zone, about one quarter inch wide. Inside this zone, except at the upper portion of the lesion, the affection presents a warty appearance, the papillæ being irregular in shape with rounded tops, separated in places by deep fissures and abrasions, and capped in places by brownish-black crusts. On pinching up the lesion with the fingers, drops of pus or sero-purulent matter appear between the papillæ. The upper portion of the lesion, as well as the whole center of the lesion inside the warty zone presents a flattened, smooth, shiny, cicatricial appearance, livid red in color, mottled by remnants of brownish-black crusts. Just below and external to this oval patch is a nearly circular patch resembling the first one, except that the whole central portion of the patch is made up of brownish-red warty excrescences, many of which are covered with dark brown crusts. The papillæ are separated from one another by deep fissures. In places where abrasions exist, the excoriated papillæ present a moist, angry appearance, and pus or seropurulent matter may be pressed out. Both patches are movable upon the underlying parts. In the livid zone surrounding the lesions there are a few miliary pustules.

Nov. 9, 1905. Under treatment with the Röntgen rays and local applications, the circular lesion on lower and outer surface of wrist has increased slightly in size. Both lesions still surrounded by purple red zones. Over the large lesion the surface is smooth, except in a few places over the lower border adjoining the smaller lesion, over which as over the smaller lesion itself there still persist warty prolongations, although greatly diminished in size.

Nov. 10, 1905. Under ether, the warty area of both lesions curetted away. Tissue found to be soft and pulpy. Came away easily. Wound washed with corrosive sublimate and treated with dry dressing.

December 18. Entire area closed in with the exception of two small areas which are red with dry surfaces on lower and outer portion of lesion. The whole is of a pale red color. Over curetted area, good, firm, smooth cicatrix. Over non-curetted area cicatrix softer and marked by minute pit-like depressions. Here and there on the border are a few narrow areas of villiform epidermic projections. From one small pustule on outer edge four days ago, blastomycetes were demonstrated under the microscope from the pus obtained therefrom. Since then no pustules seen.

December 19. Discharged, much relieved, to skin out-patient department.

. The suspicion of blastomycosis was awakened at the outset. While the affection resembled tuberculosis verrucosa to a considerable degree, the more purulent character and, as the lesion improved, the presence of cutaneous abscesses along the edge, were very striking features. Several pieces were excised and the microörganisms found in moderate quantity without difficulty, chiefly in the abscesses.

It is interesting to note that this, the first case as yet detected at the skin department of the Massachusetts General Hospital, which draws from all of New England and the British Provinces, was in an Italian subject. Dr. Hyde has called attention to the fact that several of the Chicago cases were likewise in Italians. The subject of the present paper had lived in the country when in Italy, being employed on a farm.

Pathology. — Microscopically the tissue from this case corresponds perfectly with the descriptions given by Gilchrist and Stokes, Hyde and Montgomery, and Ricketts.

The essential characteristics are the great hyperplasia of the epidermis, the presence of minute abscesses surrounded by epithelium and chronic inflammatory changes in the corium.

The epidermis sends long, branching anastomosing processes into the corium and contains many abscesses which vary in size from a collection of a few polymorphonuclear leucocytes to those just large enough to be seen in the sections with the unaided eye. The epithelium is extensively invaded by polymorphonuclear leucocytes, and there are occasional small masses of detritus, containing leucocytes, surrounded by flattened partly cornified epithelial cells. The epithelial abscesses are usually circular in cross section and well surrounded by epithelium; an occasional one is incompletely surrounded and projects into the corium. The abscesses contain chiefly polymorphonuclear leucocytes, also many eosinophiles, a few large mononuclear phagocytic cells, an occasional giant cell, a variable amount of detritus and the organisms. The giant cells are not constant, they resemble those found in tuberculosis. None were seen containing the organisms. An occasional giant cell is found in the epithelium surrounded only by a few leucocytes, an unusual picture which has been noted by Hyde and Montgomery.

The organisms, while not difficult to find, occur only in small numbers. Budding forms were found. They vary in size from seven to twelve microns and are found singly, in pairs, and in groups of three to five. The protoplasm stains with ordinary nuclear stains. With Gram's method only a few granules retain the stain. These granules stain by other methods as well, and occasionally are arranged in groups suggestive of chromatin bodies (Fig. 5). The capsules stain poorly with eosin, and with Mallory's methylene blue and eosin stain usually show as faintly pink, refractive, thick membranes. With Mallory's anilin blue stain for connective tissue fibrils (Jour. Med.

Research, XIII., No. 2) the capsules stain bright blue and the protoplasm red; and this stain is perhaps the best to use for the demonstration of the organisms in suspected tissue, because of the sharp differentiation of the capsules. The protoplasm, which is coarsely reticular and sometimes vacuolated, is usually separated from the capsules by a clear space.

The corium is markedly infiltrated. There are large collections of lymphoid and plasma cells, many polymorphonuclear leucocytes and eosinophiles. There are both small polymorphonuclear and large mononuclear eosinophiles. The latter are large cells with large round or oval nuclei and abundant protoplasm filled with small deeply staining eosinophilous granules. They are fixed in shapes indicating pronounced ameboid motion; there is no definite cell membrane. They are most numerous just below the epidermis and a few are found between the cells of the Malpighian layer. The corium is edematous in places. There are occasional small deposits of fibrin just below the epidermis. No abscesses, giant cells, or organisms were found.

Elastic tissue is readily demonstrated in the corium by means of Weigert's or Unna's stains, usually as clumps of short, thick fibers. Small amounts are occasionally found in the epidermis and epithelial abscesses. The elacin reaction was obtained in all the above places using Unna's acid orcein, polychrome blue, tannin, and Orange G method[1] — evidence of degeneration of elastic tissue according to Unna. Although occasional bulbous ends to the degenerated elastic fibers were found, nothing was seen that, using even the imagination, could be confused with the organisms. The organisms by this method stain characteristically with the Orange G and methylene blue, the first staining the capsule, the second the protoplasm. The last fact is of interest because Unna, in a recent paper, hints that the elacin bodies he found in a supposed case of cutaneous blastomycosis are what various authors have called blastomyces.[2] The conclusion is unavoidable that Unna's case was not identical with those called cutaneous blastomycosis, or, if it was, he failed to recognize the organisms.

Cultures. — On November 4 material was obtained from several miliary sized abscesses at the periphery of the lesion and placed upon glucose agar slants. To obtain the pus the skin was carefully washed with soap and water and treated with corrosive sublimate 1–1000 and alcohol. The abscesses were opened with a sterile needle and droplets of pus removed with a platinum wire for inoculation. The inoculated tubes were divided into two sets, and one kept at 37°–38° C. in the incubator, the other kept beneath the incubator, where the temperature is slightly higher than room temperature. The first signs of growth appeared on November 8 in the incubated tubes. The colonies were just visible as minute, translucent points. Fresh preparations were made and showed torula-like forms in pairs and chains. The colonies were then rubbed over the surface of the tubes and in a few days new colonies became visible. Transplants from these tubes proved negative, although various agar media were tried. Growth on the original tubes never became abundant and none of the colonies seemed to increase in size after they first became visible.

On December 12, however, growth was observed in the tubes kept at sub-incubator temperature. These colonies closely resembled young colonies of Oidium lactis and microscopically consisted of coarse, branching mycelia. The tubes in the incubator, which had shown no further growth, were now removed and placed beneath the incubator and in the course of a week showed small, lustrous, radially striated colonies which finally developed into growths similar in all respects to those first obtained upon tubes kept at sub-incubator temperature. That these mycelial growths are derived from the organisms found in the pus is proven by the results of the animal inoculations. From the above it is evident that a limited growth may take place by budding at 37°–38° C.; also, as has been repeatedly proven by others, that the vegetative form is mycelial. Attempts to convert the mycelium into the torula form by replacing the tubes into the incubator failed, though growth continued to take place very slowly. In very old cultures, bodies are found in large numbers which resemble somewhat the forms found in tissue. These bodies in cultures do not show budding, and when placed upon new media they grow by sending out mycelia. They have a well-defined membrane, which is not so thick as those about budding forms in tissue, and occasionally they are filled with large numbers of refractive spherules considered to be spores by some previous workers. We have made an attempt to discover the nature of these spherules, and thus far the results tend to the conviction that they are not spores, for the following reasons: 1. They are never found in sections of lesions. 2. When placed upon fresh media they may be seen to fuse together before the mycelial offshoot begins. 3. They are very variable in size and number. 4. Only one mycelial offshoot comes from a single sphere containing these spherules, while the spore containing spheres of the organisms of " Coccidioidal granuloma " yields many.

The cultures grow readily at room temperature upon all ordinary media, not so well, however, as at the slightly higher temperature obtained beneath the incubator. The addition of sugars does not materially influence the rate of growth. The action upon sugars has not been fully studied, but a single experiment indicates that there is no marked fermentative action. The titration method was used and the following bouillons tested: Sugar free, Maltose, Dextrose, Lactose, and Saccharose. The inoculated tubes were sealed with paraffine and allowed to grow beneath the incubator for twenty-three days. A set of control tubes were similarly sealed and kept in the same rack. At the end of this time all the inoculated tubes showed about the same amount of growth, about a third of the bouillon in each instance being filled with the fluffy, downy growth.

The tubes titrated as follows:

	Control tubes.	Inoculated tubes.
Maltose	1.4	1.7
Dextrose	1.1	1.6
Saccharose	1.1	1.3
Lactose	1.7	1.9
Sugar free	1.5	1.6

On all solid media the growth becomes closely incorporated with the medium and at first is very slightly elevated, translucent, grayish. Old cultures are white and have aerial hyphæ bearing terminal spheres filled with the spore-like bodies. Similar spheres are found attached to short, lateral offshoots. In very old glucose and glycerine agar cultures the medium becomes brown and soft. Gelatine is very slowly liquefied. In bouillon there may be either a surface or a deep growth according to the method of inoculation. The surface growth at first is grayish, translucent, later it has a white, dry, powdery upper surface. The under surface projects downwards into the bouillon as a delicate, fluffy, hemispherical mass. The deep growth forms grayish, white, thistle-down or fibrin-like spherical masses composed of radiating filaments.

Animal experiments. — Inoculation experiments were made with the pure cultures upon rabbits, guinea-pigs, and white mice. The latter proved by far the most susceptible.

Rabbits. — Three were inoculated intravenously into the ear veins. In two large rabbits, each weighing about two thousand grams, small abscesses were found at the sites of inoculation at the end of a week. The pus from these abscesses contained degenerated filaments and small numbers of budding spherical and ovoid organisms. Microscopically the lesions are inflammatory in character with numerous giant cells and much newly-formed connective tissue about necrotic foci. The organisms in the sections are found with much difficulty, as single or budding spheres. The lesions in lungs are slight and consist of inflammatory zones about thrombosed vessels. Usually one or two giant cells completely fill the lumen.

In one rabbit killed at the end of a month there were no macroscopic lesions in the lungs. Sections showed vessels containing giant cells and surrounded by a varying amount of infiltrated, newly-formed connective tissue. No organisms were found. In the second rabbit, killed at the end of a week, similar lesions were found and also occasional organisms within the giant cells. More extensive lesions and more organisms were found in a young rabbit weighing two hundred and sixty-five grams, killed eleven days after inoculation.

Guinea-pigs. — Three guinea-pigs, inoculated intraperitoneally with pure cultures, showed but slight lesions at the end of four to six weeks. Small swellings at the sites of inoculation and a few small nodules upon the peritoneal surface were the only lesions found at autopsy. None of the guinea-pigs lost weight or showed metastases. The organisms are extremely hard to find in the lesions and only within giant cells.

The lesions in the guinea-pigs consist of small abscesses surrounded by very thick walls of young connective tissue, the inner edge of which contains giant-cells. In the central mass of leucocytes there are often necrotic filaments, while occasional spherical, viable organisms are found in the giant cells. The largest lesions found were about one centimeter in diameter.

White mice. — These animals proved to be uniformly susceptible as the following inoculations prove.

Mouse I. — Inoculated, intraperitoneally, on Jan. 18, 1906, with about .5 cubic centimeters of a heavy suspension of a pure culture. Killed Feb. 7, 1906. No lesion found at the site of inoculation. In the peritoneal cavity there are a number of nodules as follows: one 8 x 5 millimeters, between stomach and spleen; one 5 x 2 millimeters, upper surface of liver; one two millimeters in diameter on left edge of liver and adherent to the diaphragm, and several miliary sized nodules in the omentum. In the right lung are two elevated, translucent, grayish nodules, each 1.5 millimeters in diameter. The two largest peritoneal nodules were opened and were found to contain pus and large masses of spheres, many of them budding, and a few thick filaments with terminal spheres and many lateral spherical offshoots. Sections of the lungs showed large numbers of organisms in pairs and singly and many in the act of budding.

Mouse III.—Inoculated, intraperitoneally, Feb. 13, 1906. Killed Feb. 19, 1906. Autopsy showed a small subcutaneous pocket of pus containing degenerated filaments and a few spheres. In the abdomen there were a few miliary sized nodules in the omentum and on the surface of the liver.

Mouse V.—Inoculated, intraperitoneally, March 5, 1906, after an apparently futile attempt to inoculate into the tail veins. Killed April 16, 1906, as it had rapidly lost weight after having given birth to a litter of young a few days previously. The only lesions found in the abdomen are a few peritoneal thickenings on the liver and spleen. The lungs, however, are extensively involved, both are voluminous and filled with nodules, one to two millimeters in diameter. The nodules are irregular in outline, translucent pink in color and closely packed, with small amounts of dark red lung tissue intervening. The thoracic glands are not enlarged. The fresh examination of crushed lung showed single and budding spheres in large numbers, many demonstrably within cells. The culture was readily recovered from the crushed tissue by streaking out on glucose agar plates. The sprouting of the spheres and the development of colonies about groups or single spheres was watched.

Mouse VI.—Inoculated, intraperitoneally, March 5, 1906, after an apparently futile attempt to inoculate into the tail veins. On April 2, 1906, it was found dead and beginning post-mortem changes indicated that death must have occurred about twelve hours previously. The abdominal lesions consisted of two slightly enlarged nodes in the pelvis, the peritoneal surfaces are negative. The lungs, however, are even more striking than those found in Mouse V.; they are greatly distended and rigid, and form casts of the chest cavities. The size and rigidity are due to closely packed white nodules, many of which have depressed red or gray centers. Fresh examination of the crushed lung shows enormous numbers of the spherical organisms in colony-like masses. Many budding forms were found and an occasional sphere with a short, filamentous offshoot which must be regarded as growth occurring after the death of the mouse.

Sections from the lungs of Mice V. and VI. show the nodules to be formed by alveoli, which are densely packed with cells, mostly large mono- and multi-nuclear cells enclosing the organisms which are present in great numbers. In the centers of the nodules the alveoli contain necrotic cells and leucocytes. The alveoli at the peripheries of the nodules have greatly thickened walls due to the presence of large mononuclear cells and polymorphonuclear leucocytes. A few organisms may be demonstrated in the alveolar walls, most easily in those just beneath the pleura.

The most striking feature of the mouse inoculations is the involvement of the lungs. In Mouse I. metastases from the peritoneal cavity to the lungs occurred within twenty days. In Mice V. and VI. the very extensive involvement of the lungs with almost no peritoneal lesions is most striking. At the time of inoculation it was believed that none of the suspension entered the veins, but if it did the amount thrown into the peritoneal cavity certainly was very many times greater. Neither of these two mice had lesions in the tails where the inoculations were attempted. The type of lesion in the lung, the filling of the alveoli with large cells and organisms with little other reaction, is a peculiar one and deserves further study.

In the above account of a case of "Cutaneous Blastomycosis" no attempt has been made to offer more than careful work sufficient to establish the etiological relationship of the organisms isolated to the disease and to permit of the correct classification among cases already reported. This we believe to be of great importance as recent publications by Unna (loc. cit.) and Krause (Monatshefte für Praktische Dermatologie, Bd. 41, Nos. 4, 5, 7, and 12) have practically denied the existence of this disease. These authors after studying single cases, which they regarded as identical with those reported in this country, have come to the conclusion that the organisms as described in lesions may be only the products of degenerated elastic tissue (Unna), or the hyaline bodies frequently found in connection with plasma cells (Krause). Krause regards the organisms to be contaminations from the air and as such to have no etiological connections with the disease.

There is likewise confusion, here and abroad, regarding the classification of those cases reported by Posadas and Wernicke, Rixford and Gilchrist, Ophüls, and D. W. Montgomery, and most commonly called Protozoic Dermatitis and Dermatitis Coccidioides. As the result of a study made by one of us in 1904, from a case in Boston still under observation,[3] we believe with Dr. D. W. Montgomery that the organisms from the two types of cases, the "Blastomycotic" and "Coccidioidal" are distinct varieties. It is certain that each organism has a number of constant, independent characteristics, brought out by animal inoculations, which cannot be duplicated in the other. At present the weight of opinion seems towards regarding the cases of "Blastomycotic" and "Coccidioidal" infection as identical.[4] The question is still an open one. Our own cases are certainly very different clinically, and the organisms recovered show many striking differences. More work is necessary to satisfactorily settle this question. For the present we wish to emphasize the necessity for careful studies upon the organisms by means of animal experiments, for it is by this method only that the more striking differences between the two types may be demonstrated. Cultural methods as applied to bacteria have not been of value to us.

We believe that this case is identical with most of the cases in the larger group reported as "Cutaneous Blastomycosis," chiefly by Gilchrist, Hyde and Montgomery, and Ricketts, in distinction from the smaller group of cases, of which there have been nine, and which have been called "Protozoic" or "Coccidioidal."

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DESCRIPTION OF PLATES.

PLATE XVI.

FIG. 1. Section of the lesion near the periphery of the growth from tissue excised Nov. 10, 1906. x 100 diameters. Methylene blue and eosin stain. Shows two epithelial abscesses, the characteristic epithelial invasion of the corium and a giant cell in the epithelium as described by Hyde and Montgomery, unsurrounded by inflammatory tissue.

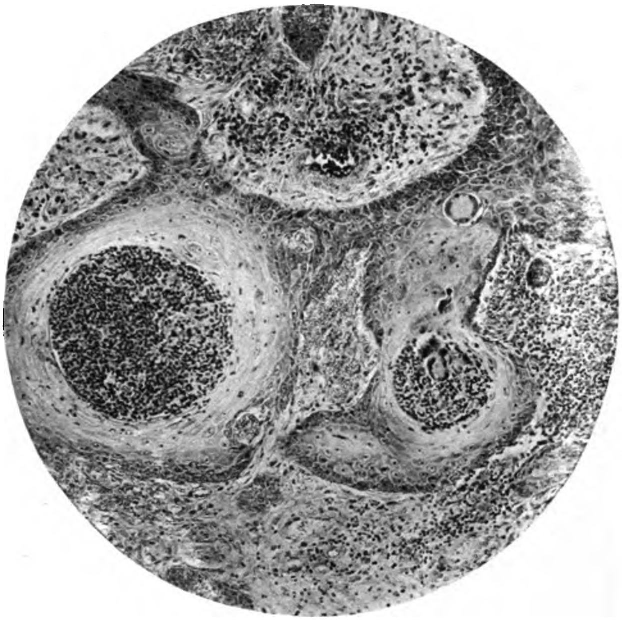
FIG. 2. High power x 1,000 diameters of the larger abscess shown in figure one, showing the organisms. Methylene blue and eosin stain. The larger of the two spheres has a budding process not quite in the plane of focus.

PLATE XVII.

FIG. 3. Pure cultures on potato. Natural size. Shows two types of growth on solid media. The spiculated colonies occur infrequently. The spicules are made up of parallelly arranged filaments.

FIG. 4. Section of lung, Mouse V., showing numbers and variation in sizes of the organisms. x 1,000 diameters. Mallory's connective tissue stain.

FIG. 5. Same as Fig. 4, different field. Note large dividing organism with four deeply staining granules resembling chromatin. Other budding organisms are in this field.



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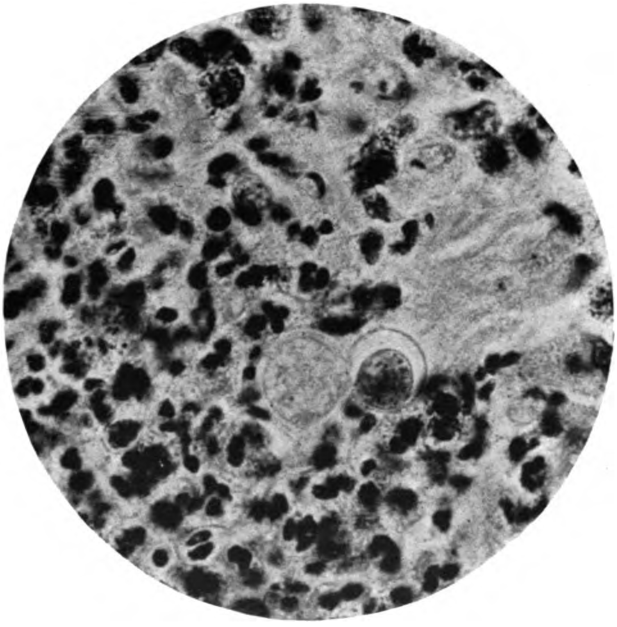

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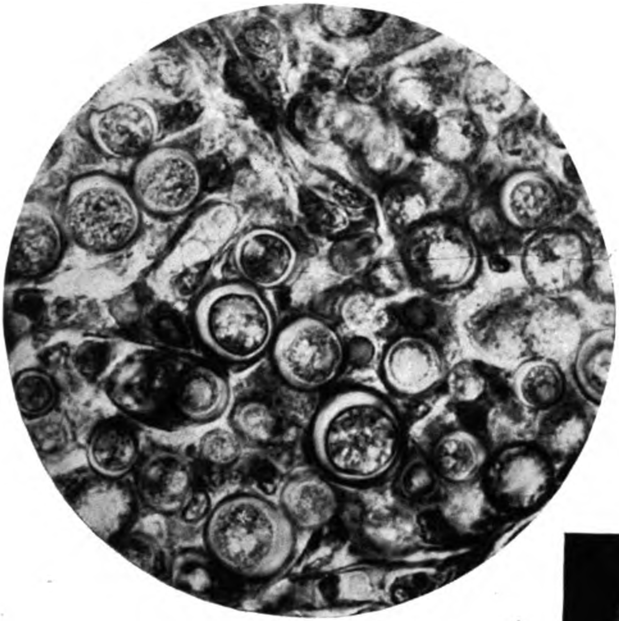

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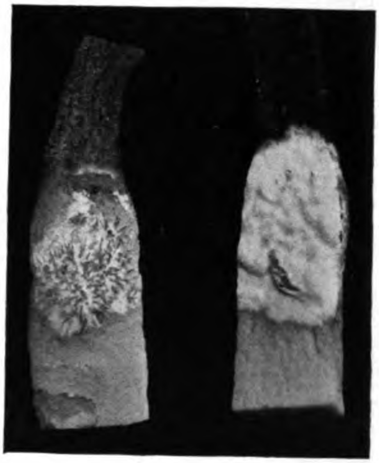

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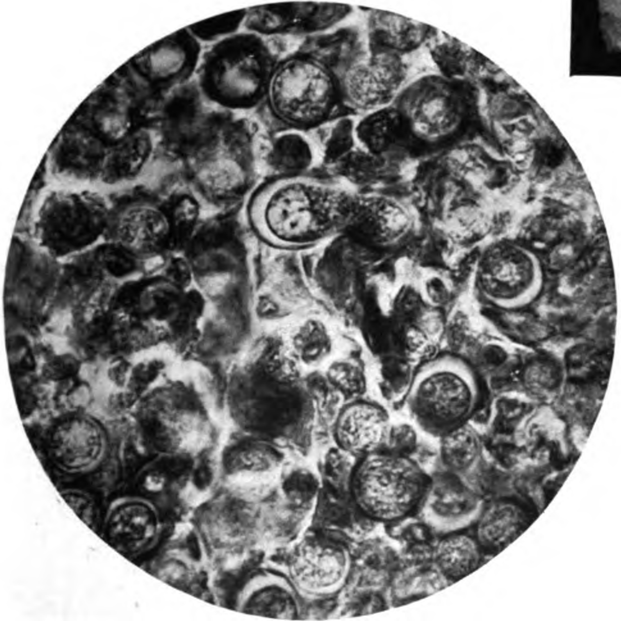

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A NEW TOXIC ACTION OF HORSE SERUM.*

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Horse serum, when injected into a normal guinea-pig, apparently causes no more inconvenience than the injection of so much sterile salt solution. However, when horse serum is injected into a used guinea-pig it causes the sudden death of the animal. By "used" guinea-pig is meant one that has recovered from an injection of the toxine-antitoxin mixture used in testing the potency of diphtheria antitoxin. This phenomenon has long been known to some of the manufacturers of diphtheria antitoxin; but it first occurred to one of us (Anderson) that a study of the phenomenon might throw light upon the cause of sudden death following the injection of horse serum into man.

In a certain proportion of cases the injection of horse serum into man is followed by urticarial eruptions, joint pains, fever, swelling of the lymph nodes, edema and albuminuria. This reaction, which appears after an incubation period of eight to thirteen days, has been termed by von Pirquet and Schick the "serum disease."

In exceptional instances sudden death has followed an injection of horse serum in man.

We have shown that ordinarily horse serum is a comparatively bland and harmless substance, but certain animals may be rendered so susceptible that an injection of horse serum may produce sudden death or severe symptoms. For example, if a guinea-pig is injected with a small quantity, say $\frac{1}{250}$ cubic centimeter of horse serum and after the expiration of a certain interval is again injected with horse serum the result will probably be fatal. The first injection of horse serum has sensitized the animal in such a way as to render it very susceptible. It is probable that when the guinea-pig is injected with the first, or sensitizing, quantity of serum the strange proteid contained in the horse serum develops in the body of the guinea-pig "anti-bodies" which, when brought into contact with more horse serum given at a second injection, cause the toxic action.

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* Read before the American Association of Pathologists and Bacteriologists, Baltimore, May 18, 1906.

A certain time is necessary to elapse between the first and second injection of horse serum before this toxic action is able to manifest itself. This "period of incubation" is about ten days and corresponds suggestively with the period of incubation of the serum disease, which von Pirquet and Schick place at eight to thirteen days.

While at first we thought that diphtheria antitoxin had some relation to this action, we are able to state positively that it has nothing whatever to do with the poisonous action of horse serum; further, that diphtheria antitoxin in itself is absolutely harmless. The toxic action which we have studied is caused by a principle in normal horse serum and is entirely independent of the antitoxic properties of the serum.

The fact that the toxic action of horse serum may be developed by exceedingly small quantities of the serum and that very small quantities are sufficient to produce symptoms or death upon a second injection, and the further fact that an immunity may readily be produced against the toxic action, a priori places both the sensitizing and the toxic principle in the horse serum in the "haptin group" of substances in the sense used by Ehrlich.

We made many control experiments, proving that horse serum, either normal or antitoxic, when injected intraperitoneally in large or in small quantities into normal guinea-pigs causes no symptoms. When injected subcutaneously there may result at most a local reaction indicated by edema and induration at the site of injection.

We have injected many guinea-pigs with antitoxic serum in the routine examination of diphtheria antitoxin in accordance with the law (of July 1, 1902), and have never observed any untoward effects except in cases of bacterial or toxic contamination.

Characteristic symptoms are produced by horse serum when injected into a susceptible guinea-pig. The symptoms are apparently the same whether the injection is made subcutaneously or into the peritoneal cavity, or whether normal or antitoxic horse serum is used. In five or ten minutes after injection the pig manifests indications of respiratory embarrassment by scratching at the mouth, coughing, and sometimes by spasmodic, rapid, or irregular breathing; the pig becomes restless and agitated. This stage of exhilaration is soon followed by one of paresis or complete paralysis. The pig is unable to stand, or, if it attempts to move, falls upon its side; when taken up it is limp. Spasmodic, jerky, and convulsive movements now supervene.

Pigs in the stage of complete paralysis may fully recover, but usually convulsions appear and are almost invariably a forerunner of death. Symptoms appear about ten minutes after the injection has been given; occasionally in pigs not very susceptible they are delayed thirty to forty-five minutes. Only in one or two instances of the several hundred pigs which we have observed have the symptoms developed after one hour. Pigs developing symptoms as late as this are not very susceptible and do not die. The symptoms do not always follow in the order given. Death usually occurs within an hour and frequently in less than thirty minutes.

Judging from the symptoms produced by the injection of horse serum into a susceptible guinea-pig, we assumed that the poison acted upon the nervous system. Autopsies done immediately after the death of the guinea-pigs invariably showed that the heart continued to beat long after respiration had ceased. In some instances the heart after exposure continues to beat a full hour. This would seem to indicate that we are dealing with a poison which caused death through the nervous control of the respiration, and the following experiment shows that this effect certainly is not local:

G.P. No. 4,526. — Six cubic centimeters antitoxic horse serum (Natl. VIII., 17) injected into the peritoneal cavity. Dead in thirty minutes.

[Previous treatment: Seventeen days prior, inoculated subcutaneously with .19 cubic centimeters toxine No. 7 + 1 unit antitoxic horse serum (Standard B27).]

Immediately after the death of this guinea-pig the phrenic nerve was exposed and stimulated high up, causing contractions of the diaphragm, both upon making and breaking the galvanic current. The contractions were also caused with the induced current. The contractions of the diaphragm, caused by stimulating the phrenic nerve in this way, were produced with a weaker current than that required to cause similar contractions in a normal guinea-pig. We are indebted to our colleague, Dr. Reid Hunt, for assistance in this part of our work.

It occurred to us that possibly diphtheria antitoxic serum may contain free toxone, which, when injected into a "used" guinea-pig, might produce acute effects upon the nervous system of such a pig, which, on account of the previous effects of the diphtheria toxine, had been rendered peculiarly susceptible. It is well known that toxone does not cause the acute death of a guinea-pig, but only produces local edema and late paralysis. This fact, first shown by Ehrlich, may readily be demonstrated by inoculating guinea-pigs with toxine and antitoxine mixed in such proportions that the toxine is but partly neutralized. We were further led to believe that the poisonous effects of horse serum upon a treated guinea-pig might be a manifestation of the toxone because the symptoms so plainly point to the nervous system. The pigs almost invariably show paresis or paralysis.

The fact that neither toxone nor any other constituent in the toxine plays any rôle in the poisonous action of horse serum is demonstrated in the following example:

G.P. No. 4,161. — 0.1 cubic centimeter toxine No. 15 (MLD = .01) was injected into the peritoneal cavity. No symptoms. Completely paralyzed at the time, resulting from previous treatment.

[Previous treatment: Thirty-one days prior, inoculated subcutaneously with 0.22 cubic centimeter toxine No. 7 + $\frac{1}{210}$ cubic centimeter diphtheria antitoxic serum (Mulford's 942).]

One hour fifty minutes later this same pig was inoculated with six cubic centimeters diphtheria antitoxic serum (P.D. & Co. 08022) into the peritoneal cavity. Dead in ten minutes.

It is well known that overpowering doses of toxine injected either subcutaneously or into the peritoneal cavity of a guinea-pig require about twenty hours to kill the animal. These large doses of toxine do not cause immediate symptoms.

It occurred to us, however, that while toxine alone may not have this power, nevertheless when it is mixed with antitoxic horse serum and when large doses of the mixture are injected into a guinea-pig there may be a sufficient liberation of toxone or other poisonous substances capable of producing symptoms and sudden death.

The following mixture of toxine and antitoxin represents these two substances in the proportion of the L^0 dose.

G.P. No. 4,483. — 1.6 cubic centimeters toxine No. 7 + 11.5 units antitoxic serum (Standard B27) injected subcutaneously. No symptoms.

Eighteen days later six cubic centimeters antitoxic horse serum (Natl. VIII., 17) injected into the peritoneal cavity of the same pig. Death thirty minutes.

In a similar way the effects of mixing large doses of the diphtheria toxine with normal horse serum were tested as follows:

G.P. No. 40. — Six cubic centimeters normal horse serum (roan) + 0.3 cubic centimeter diphtheria toxine No. 5 mixed in vitro and injected into the peritoneal cavity. No immediate symptoms. Died two days later from the effects of the toxine.

When this work was first undertaken it was thought to have a bearing upon sudden death following the injection of horse serum in children suffering with diphtheria. The work was begun in order to discover, if possible, the cause of this accident. We thought that possibly by injecting small quantities of a virulent culture of the Klebs-Löffler bacillus into guinea-pigs, in that way imitating mild cases of diphtheria, some of these pigs could be rendered susceptible to subsequent inoculations of antitoxic horse serum. This, however, is not the case for guinea-pigs. A large number of guinea-pigs was treated with Klebs-Löffler bacilli, sufficient to cause slight symptoms, and at varying intervals of time were again inoculated with large quantities of antitoxic horse serum. In no instance did the guinea-pig show any ill effects from this latter treatment.

For the same reason that guinea-pigs were treated with the cultures of the bacillus diphtheriæ we also attempted to render them susceptible by the injection of small quantities of diphtheria toxine. On account of the great importance of this subject a large number of guinea-pigs was used to demonstrate the rôle which the diphtheria toxine may play in the subsequent action of horse serum. Our experiments demonstrate conclusively that diphtheria toxine alone plays absolutely no rôle in rendering guinea-pigs susceptible to the toxic action of horse serum, for in no instance did the guinea-pig show the slightest effects when subsequently injected with large quantities of serum.

Before we realized that the diphtheria toxine or antitoxin played no rôle in rendering guinea-pigs susceptible to subsequent injections of horse serum we prepared a series of guinea-pigs in order to eliminate the antitoxin as a factor. These pigs were injected subcutaneously with a mixture of one cubic centimeter of normal horse serum and a small quantity of diphtheria toxine, slightly less than a minimal lethal dose. This was done for comparison with the susceptibility produced by the toxine-antitoxin mixtures. Example:

G.P. No. 296. — Six cubic centimeters normal horse serum (roan) injected into the peritoneal cavity. Dead in sixty minutes.

[Previous treatment: Seventy-one days prior, inoculated subcutaneously with 0.002 cubic centimeter toxine No. 7 + 1 cubic centimeter normal horse serum (Sam).]

Having shown that diphtheria plays no rôle in this action, we next excluded the antitoxin by treating a number of guinea-pigs, giving them at both the first and second injections fresh normal horse serum, as shown by the following example:

G.P. No. 101. — Three cubic centimeters normal horse serum (roan) injected into the peritoneal cavity. Dead in thirty-five minutes.

[Previous treatment: Fifty-five days prior, inoculated subcutaneously with $\frac{1}{250}$ cubic centimeter normal horse serum (Jane).]

Whether the poisonous substance in horse serum is specific may be determined in several ways. We first endeavored to determine whether guinea-pigs treated with horse serum are susceptible to the serums of other animals; and, secondly, whether guinea-pigs treated with the serums of other animals are susceptible to subsequent injections of horse serum. The following is a list of animals treated with horse serum and subsequently tested with the blood serum of other animals. It will be noticed that in this list some of the guinea-pigs were treated with donkey serum instead of horse serum, but as the donkey serum in our hands has shown itself to act the same as horse serum we have used them interchangeably.

Table showing the effect of alien serums upon guinea-pigs sensitized with horse serum.

No. of Guinea-pig.	Kind and Amount of Normal Serum.	Previous Treatment.		Result.
		Days Prior.	Inoculated Subcutaneously with —	
178.	Cattle, 6 cc.	34	$\frac{1}{250}$ cc. donkey serum	Symptoms, recovered.
149.	" "	41	0.22 cc. toxine No. 7 + $\frac{1}{300}$ cc. horse serum (Stearns, 1351)	" "
180.	Hog, 6 cc..........	34	$\frac{1}{250}$ cc. donkey serum.................	No symptons.
138.	" "	41	0.22 cc. toxine No. 7 + $\frac{1}{300}$ cc. horse serum (Stearns, 1351)	Symptoms, recovered.
4,743.	" 1 cc..	41	0.139 cc. toxine No. 5 + $\frac{1}{350}$ cc. horse serum (Alex., 192)..................	" "
179.	Sheep, 6 cc.	34	$\frac{1}{250}$ cc. donkey serum	" "
152.	" "	41	0.22 cc. toxine No. 7 + $\frac{1}{300}$ cc. horse serum (Stearns, 1351)	" "
177.	Cat, 6 cc..........	34	$\frac{1}{250}$ cc. donkey serum	" "
124.	" "	41	0.22 cc. toxine No. 7 + $\frac{1}{300}$ cc. horse serum (Stearns, 1351)	No symptoms.
4,744.	" 1 cc...........	41	0.139 cc. toxine No. 5 + $\frac{1}{350}$ cc. horse serum (Alex., 192)	Symptoms, recovered.
121.	Rabbit, 6 cc.	41	0.22 cc. toxine No. 7 + $\frac{1}{300}$ cc. horse serum (Stearns, 1351)	" "
4,380.	" "	19	0.22 cc. toxine No. 7 + $\frac{1}{250}$ cc. horse serum (Natl. V., 10)	" "
4,374.	" "	19	0.22 cc. toxine No. 7 + $\frac{1}{250}$ cc. horse serum (Natl. V., 7)	" "
153.	Dog, 6 cc..........	41	0.22 cc. toxine No. 7 + $\frac{1}{300}$ cc. horse serum (Stearns, 1351)	" "
4,379.	" "	19	0.22 cc. toxine No. 7 + $\frac{1}{250}$ cc. horse serum (Natl. V., 10)................	" "
4,742.	" 1 cc..........	41	0.139 cc. toxine No. 7 + $\frac{1}{350}$ cc. horse serum (Alex., 192)	" "
4,375.	Guinea-pig, 3 cc...	19	0.22 cc. toxine No. 7 + $\frac{1}{250}$ cc. horse serum (Natl. V., 7).................	No symptoms.

As the above series of guinea-pigs previously treated with horse serum showed unmistakable susceptibility to subsequent injections of the blood serum of other animals, we are justified in saying that the action is not specific; but there is a very evident quantitative difference. It will be noticed that none of these guinea-pigs died, and it was a matter of observation that, for the most part, they had mild though characteristic symptoms.

We next took up the question as to whether a guinea-pig may be sensitized to horse serum with the blood serum of animals other than the horse. The following is a list of guinea-pigs which had been previously treated by the subcutaneous inoculation of small quantities ($\frac{1}{250}$ cubic centimeter) of blood serum from the rabbit, rat, donkey, cat, dog, hog, sheep, chicken, cattle, and man. Each pig was subsequently injected with six cubic centimeters antitoxic horse serum (Natl. IX., 17) into the peritoneal cavity.

Table showing the results of injecting six cubic centimeters of antitoxic horse serum (Natl. IX., 17) into the peritoneal cavity of guinea-pigs treated from twenty-five to thirty-four days prior with $\frac{1}{250}$ cubic centimeters of the blood serum of other animals.

No. of Guinea-pig.	Previous Treatment.		Result.
	Kind of Serum.	Days Prior.	
171	Rabbit $\frac{1}{250}$ cc.	25	No symptoms.
172	" " "	34	" "
174	" " "	34	Symptoms, recovered.
167	Rat " "	25	No symptoms.
168	" " "	34	" "
169	" " "	34	" "
170	" " "	34	" "
176	Donkey " "	25	" "
191	Cat " "	25	" "
192	" " "	34	Symptoms, recovered.
193	" " "	34	Symptoms (?) recovered.
214	Dog " "	26	No symptoms.
215	" " "	35	" "
212	" " "	35	Symptoms, recovered.
185	Hog " "	30	No symptoms.
186	" " "	30	Symptoms, recovered.
182	Sheep " "	30	" "
184	" " "	30	No symptoms.
196	Chicken " "	26	Symptoms, recovered.
197	" " "	26	" "
206	Cattle " "	31	No symptoms.
207	" " "	31	" "
210	" " "	31	" "
201	Man " "	31	Symptoms, recovered.
202	" " "	31	No symptoms.

We think it is plain from the above that this action is not strictly specific for horse serum; that is, guinea-pigs treated with horse serum are rendered somewhat susceptible to the subsequent injection of the serum of certain other species, and that guinea-pigs treated with the serum of other animals are slightly sensitive to the toxic action of horse serum.

In other words, there is plainly a distinct quantitative difference, both in the results of the power of horse serum to render guinea-pigs susceptible to other serums and in its toxic action upon guinea-pigs sensitized by other serums.

We now know that guinea-pigs previously treated with small injections of horse serum become very susceptible to subsequent injections of horse serum, but only slightly susceptible to the blood serums of other animals. It became important to determine whether guinea-pigs treated with the serums of other animals (dog, hog, rat, cattle, sheep, cat) become as highly sensitized to a second injection of the homologous serum.

A series of guinea-pigs was sensitized with $\frac{1}{250}$ cubic centimeter of the blood of various animals. Subsequently, the pigs were injected with horse serum, to which they either did not respond or, after characteristic symptoms, recovered. These guinea-pigs were finally injected with large quantities of the same kind of serum as that used at the first injection. Example:

G.P. No. 211.—Four cubic centimeters normal *horse* serum (roan) injected into the peritoneal cavity. Symptoms.
[Previous treatment: Fifty-six days prior, inoculated subcutaneously with $\frac{1}{250}$ cubic centimeter *dog* serum.]
One day later six cubic centimeters *dog* serum injected into the peritoneal cavity. Dead in thirty-five minutes.

We found that guinea-pigs treated with the serums of various animals and subsequently injected are much more susceptible to homologous serums than to heterologous serums. We have here another indication that this toxic action of blood serum is quantitatively specific. We are now studying this with the serums of other animals more in detail, and the results will be reported at another time.

So much work has recently been done indicating that the toxic property of blood serum is closely allied with its hemolytic action that we made some experiments in order to determine what connection exists between the hemolytic and toxic action of horse serum upon guinea-pigs. As a result of these studies we believe that we have shown very clearly that blood serum may contain an acute poison entirely independent of any hemolytic action.

We found horse serum to have practically no hemolytic action upon the corpuscles of the guinea-pig. This applies with equal force to antitoxic horse serum as to normal horse serum.

As a further corroboration we may state that autopsies upon guinea-pigs which have died from the results of the injections of horse serum showed no unusual effects, indicating that hemolysis took place within the body.

Further, horse serum heated to 60° C. for six hours is as toxic as unheated serum.

The precipitins are so specific and so closely related to the cytotoxins that it naturally occurred to us to determine what relation there may exist between the toxic power of the blood serum of the horse and the guinea-pig and the power of these two serums to precipitate each other.

If we inject horse serum several times into a guinea-pig we would expect to find that the blood serum of a guinea-pig so treated would have a power of precipitating horse serum. This we found to be the case; but we could not find that the injection of a single small dose of horse serum into the guinea-pig developed this power in the guinea-pig's blood serum. We might, however, conjecture that the union of the two serums under the conditions named causes a precipitation which is not visible, or causes a chemical union between two highly organized albuminous substances having toxic properties.

We made some tests to determine whether a guinea-pig previously treated with one small injection of horse serum developed precipitins.

One cubic centimeter of horse serum, both normal and antitoxic, in dilution of 1:21 was placed in small tubes and 0.1 cubic centimeter of a sensitized guinea-pig serum added to each tube as shown in the following table. They were shaken and placed in the incubator and results read after twenty-four hours.

G.P. No. 4,617. — [Previous treatment: Thirty-five days prior, inoculated subcutaneously with 0.19 cubic centimeter toxine No. 7 + 1 unit horse serum (Standard B27).]

G.P. No. 4,510. — [Previous treatment: Same.]

1 cc. Horse Serum (1: 21).	0.1 cc. Normal Guinea-pig Serum.		0.1 cc. Treated Guinea-pig Serum.	
	Centrifugation.	Coagulation.	Guinea-pig 4,517.	Guinea-pig 4,510.
Normal horse serum (Sam)	0	0	0	0
" " " (Jane)	0	0	0	0
" " " (S.P.)	0	0	0	0
" " " (donkey) ...	0	0	0	0
Antitoxic serum (Natl. VIII., 17) .	0	0	0	0
" " (" V., 14)....	0	0	0	0
" " (" IX., 17) ...	+ (?)	0	0	0
" " (Stearns, 1,110)..	0	0	0	0
" " (" 1,429)..	0	0	0	0

It would therefore appear that guinea-pigs which have previously been inoculated with one injection of a very small amount of horse serum sufficient to render them highly susceptible to a second injection of horse serum do not contain sufficient precipitin in their blood serum to cause a visible precipitation when added to horse serum.

The following studies show still more clearly that the specific precipitins have nothing to do with this toxic reaction.

We prepared guinea-pigs by injecting them many times at short intervals with horse serum so as to develop a high degree of precipitin in their blood serum against horse serum. The blood serum of guinea-pigs so prepared was then added to horse serum in the proportions of about one of the former to two of the latter, and allowed to stand until the precipitating action was complete. This guinea-pig serum was so active that very large quantities of the precipitum settled to the bottom of the test-tubes. The mixture was now filtered so as to separate the precipitum entirely and the filtrate injected into sensitized guinea-pigs and found to be quite as toxic as untreated horse serum.

The results of this part of our work are strengthened by the conclusions of von Pirquet and Schick, who found that the antibodies, whatever they may be, producing symptoms of the "serum disease" are not identical with the precipitins. They found, for instance, that the symptoms of the serum disease appear within eight to thirteen days following the first injection of horse serum into man, whereas it requires about three weeks for precipitins to appear in the blood of children after the injection of horse serum.

The formation of precipitins does not take place as readily in man following the injection of horse serum as it does in rabbits. In fact, von Pirquet and Schick found that sometimes, even after the injection of two hundred cubic centimeters, there was no production of precipitins, and further, that the length of time that the precipitin reaction remains in the blood of man following the injection is variable. It sometimes disappears in the fourth week and sometimes is still markedly present in the ninth week.

Finally, we know that the precipitins are strictly specific, whereas the toxic action is only quantitatively so.

One of the first questions which required solution was whether the toxic substance in blood serum is thermolabile.

We found that while a temperature of 60° C. continued for six hours has practically no influence upon the toxic substance in horse serum, the toxicity is entirely destroyed by a temperature of 100° C. for fifteen minutes.

The close-grained pores of a Pasteur-Chamberland filter B do not retain the toxic principle, as the filtrate was as poisonous as the unfiltered serum.

We have on hand a large quantity of dried antitoxic horse serum which has been prepared and preserved under special conditions for the preparation of the standard antitoxic unit. The serum was carefully dried under bacteriologic precautions at a temperature below 37° C. Such dried and redissolved serum retains its toxic property.

We made several experiments to determine whether precipitation by ammonium sulphate and subsequent dialysis to remove the sulphate and other inorganic salts would also remove the toxic principle. For this purpose we used antitoxic horse serum prepared and dialized in accordance with Gibson's method. We found that precipitation and dialysis do not remove the toxic principle.

Various substances of widely different chemical nature were added to horse serum in order to see whether any of them had the power of precipitating, oxidizing, or neutralizing the toxic property of the serum. The following substances were used: Succinic acid peroxide (alphozone), butyric acid, hydrogen dioxide, permanganate of potash, alcohol, ammonium sulphate, and citrate of soda, none of which seemed to have any effect upon this toxic action.

As much of this work was done with horse serum preserved by means of chloroform and trikresol (0.4 per cent), we made a control experiment, using horse serum as fresh as possible without the addition of any substance, and found that the addition of the preservative had no effect upon the poisonous principle.

At one time in our work it appeared to us that the toxic properties of horse serum gradually diminished in toxicity with the age of the serum. It was, however, soon proved that serums eight years old may be as poisonous as those freshly drawn.

Normal horse serum was exposed to the action of X-rays, forty amperes, four inches from the tube for forty minutes, then injected into a susceptible guinea-pig. It was found as poisonous as horse serum not so treated.

In view of the practical importance in serum therapy of eliminating or neutralizing this toxic action we devoted much time to this phase of our work.

That the guinea-pig remains susceptible to the toxic action of horse serum a very long time is indicated in the following experiment, in which one hundred and sixty days elapsed between the first treatment and the second injection. How much longer this action may continue will be ascertained and reported upon at a subsequent time. Example:

G. P. No. 3,827. — Six cubic centimeters antitoxic horse serum (Natl. VIII., 18) injected into the peritoneal cavity. Dead in thirty-three minutes.

[Previous treatment: One hundred and sixty days prior, inoculated subcutaneously with 0.22 cubic centimeter toxine No. 7 + $\frac{1}{850}$ cubic centimeter antitoxic horse serum (Jordan, No. 201).]

A large part of our work was done upon guinea-pigs which had been used to measure the strength of antitoxic serums. As the strength of diphtheria antitoxin runs between two hundred and fifty and one thousand units to the cubic centimeter, most of our guinea-pigs were therefore rendered susceptible by doses of horse serum varying from $\frac{1}{250}$ to $\frac{1}{1000}$ cubic centimeter. It became interesting to know how small a quantity of horse serum injected into a healthy guinea-pig would render it susceptible to the toxic action of horse serum. In one instance a guinea-pig which had received only $\frac{1}{1000000}$ of a cubic centimeter at the first injection was susceptible to a subsequent injection of the horse serum.

One-tenth of a cubic centimeter of horse serum injected into the peritoneal cavity of a susceptible guinea-pig is sufficient to cause the death of the animal, whereas the same quantity subcutaneously causes symptoms.

Before we learned that the antitoxin contained in the blood serum of the horse plays no part in rendering guinea-pigs susceptible to subsequent inoculations of horse serum we planned and carried out an extensive series of experiments to determine just what relation the susceptibility of guinea-pigs bears to the antitoxic potency of the horse serum injected. One series of guinea-pigs was injected subcutaneously with one unit of an antitoxic horse serum containing two hundred and fifty units to the cubic centimeter. This series, therefore, received $\frac{1}{250}$ cubic centimeter of horse serum. The second series was treated subcutaneously with two hundred and fifty units; that is, the guinea-pig received one cubic centimeter of the horse serum. The third series of guinea-pigs was treated with one thousand units; that is, each guinea-pig received four cubic centimeters of the antitoxic horse serum. At varying intervals following these injections the pigs were given a second injection of horse serum in order to determine their susceptibility.

We found from these three series that guinea-pigs receiving four cubic centimeters of antitoxic horse serum are apparently not quite so susceptible as those receiving the smaller amounts ($\frac{1}{250}$ cubic centimeter and one cubic centimeter); also, that those which received one cubic centimeter are apparently not quite so susceptible as those which received only $\frac{1}{250}$ cubic centimeter of the horse serum. We know that it is the quantity of horse serum, and not the number of units contained in the serum, which plays the important rôle. If this relation of the amount of serum is true, we presume that animals receiving large quantities of horse serum for the first injection are less susceptible because they are rendered slightly immune, just as animals can be immunized against this action by daily repeated inoculations. When large quantities are given at the first injection, the horse serum is probably absorbed very slowly and acts somewhat in the same manner.

We also believe that guinea-pigs are not rendered quite as susceptible by first injections of horse serum as by injections of the toxine-antitoxin mixture.

Our work seems to indicate that the substance which renders the guinea-pigs susceptible to a second injection of horse serum is not free in the blood serum of the guinea-pig in the same sense that the free receptors are in diphtheria antitoxic serum.

From the fact that a sensitizing substance does not appear to be free in the blood of the guinea-pig and from the further fact that we were unable to transfer the immunity, we might infer that this reaction may not reside so much in the blood serum as in the cells of the body.

Guinea-pigs may be immunized against this toxic substance, following the lines of active immunity produced by repeated inoculations of bacterial toxins. For instance, a guinea-pig that has received an injection daily for ten days of small quantities (one cubic centimeter) of horse serum develops a high degree of immunity to subsequent injections of horse serum. Guinea-pigs that have received more than ten inoculations on ten successive days develop a still higher degree of immunity.

As a normal untreated guinea-pig is entirely insusceptible to the toxic substances found in horse serum, it naturally occurred to us to try to ascertain whether the blood serum or the juices from the various organs of such a normal guinea-pig had a restraining, neutralizing or immunizing action when mixed with horse serum and the mixture inoculated into susceptible guinea-pigs.

We found that neither the blood serum nor the organs of a normal guinea-pig have any appreciable neutralizing action upon the toxicity of horse serum when injected into a sensitized guinea-pig.

Further, we found that repeated injections of horse serum into a guinea-pig produces a certain degree of immunity to the subsequent action of horse serum, and it naturally occurred to us to try whether such guinea-pigs contained in their body juices an immunizing substance in sufficient quantity to neutralize the poisonous substance found in horse serum. We were unable by such means to transfer the immunity.

It therefore appears that the "immune bodies," if such exist against the toxic action of horse serum, are not free in the blood and body juices, contrary to the case in diphtheria.

Our work up to this point seemed to carry us very far away from an explanation of the cause of sudden death in man following the injection of horse serum. A thorough study of the literature upon this subject indicates that almost all the unfortunate cases of this kind have probably followed the first injection of horse serum. If, therefore, man may be sensitized to a toxic principle in horse serum it must be by some other means than a previous injection of horse serum. We will see further on that von Pirquet and Schick, in their studies on the serum disease, have actually injected children at intervals of several weeks without any such serious effect as we have found in guinea-pigs.

In casting about for another explanation it occurred to us that perhaps this action may take place through eating horse meat. We therefore carried on a number of feeding experiments upon guinea-pigs. We first fed five guinea-pigs with normal horse serum by means of a stomach tube.

Only one of these five pigs showed definite and characteristic symptoms following the intrastomachal administration of the horse serum. This pig (No. 115), which was sensitive to the subsequent injections of horse serum, had received ten feedings or five cubic centimeters each of normal horse serum daily. In view of the fact, however, that exceedingly small quantities ($\frac{1}{1000000}$ cubic centimeter) of horse serum are sometimes sufficient to sensitize a guinea-pig, it seemed to us that feeding animals with a stomach tube might cause slight abrasions resulting in absorption, so that pigs fed in this manner might really be sensitized through minute wounds of the mucous membrane.

We therefore used another series of pigs and fed the animals without using artificial means. As guinea-pigs will not drink horse serum, we found it necessary to dry the serum, reduce it to powder and then mix it with ground carrots. In this way they took the serum unknowingly with their food. By this means definite amounts of the horse serum may be given and the quantity taken by each animal accurately controlled. Half a gram of the dried normal horse serum was thus fed to each pig, which was afterwards tested for susceptibility.

Of the nine pigs so fed, eight reacted to subsequent injections of horse serum, one of the eight dying.

After demonstrating the possibility of rendering guinea-pigs susceptible by feeding them with horse serum we were able to demonstrate in a subsequent series that guinea-pigs may likewise be rendered susceptible by feeding them with horse meat. Four grams of dried ground horse meat were mixed with fresh carrots and fed to the pigs daily. Almost all the pigs placed upon this diet did poorly; their coats became shaggy and the animals lost weight.

The fact that guinea-pigs may be rendered susceptible by the feeding of strange proteid matter opens an interesting question as to whether sensitized guinea-pigs may also be poisoned by feeding with the same serum. If man can be sensitized in a similar way by the eating of certain proteid substances it may throw light upon those interesting and obscure idiosyncrasies in which the eating of fish and other sea food by certain individuals habitually causes sudden and sometimes serious symptoms.

As some of our susceptible female pigs had young, we embraced the opportunity of determining whether the susceptibility to the toxic effects of horse serum may be transmitted from the mother guinea-pig to her young. We tested ten young pigs born of five susceptible mothers. These young pigs were injected with one cubic centimeter of normal horse serum either subcutaneously or intraperitoneally, followed in every case by the development of characteristic symptoms. The young pigs in some instances were found more susceptible than the mother. Example:

Mother G.P. No. 14. — Six cubic centimeters normal horse serum (roan) injected into the peritoneal cavity. Symptoms, recovered.

[Previous treatment: Ninety-four days prior, inoculated subcutaneously with $\frac{1}{250}$ cubic centimeter antitoxic horse serum (Natl. IX., 18).]

The progeny of the above guinea-pig, when five days old, was tested as follows:

G.P. No. 14A. — One cubic centimeter normal horse serum (roan) injected into the peritoneal cavity. Dead in thirty minutes.
G.P. No. 14B. — Same. Dead in thirty minutes.
G.P. No. 14C. — Same. Dead in thirty-five minutes.

These results were controlled upon young guinea-pigs of the same age born of untreated mothers. In some of the controls the amount of serum was doubled. In none of them did there develop the slightest reaction.

These results upon the hereditary transmission of the susceptibility to the poisonous action of horse serum in guinea-pigs may throw light upon the well-known inherited tendency to tuberculosis in children born of a tuberculous parent.

There are certain analogies between the action of tuberculosis and horse serum. Both produce a hypersensitiveness and also a certain degree of immunity. Now that we have proved that this hypersensitiveness in the case of horse serum may be transmitted hereditarily in guinea-pigs, may it not throw light upon the fact that tuberculosis "runs in families"? While there are several recorded instances demonstrating that immunity to certain infectious diseases may be transmitted from a mother to her young, this is, so far as we know, the first recorded instance in which hypersensitiveness has been experimentally shown to be hereditarily transmitted from a mother to her young.

It may be that man cannot be sensitized in the same way that we have shown is the case with guinea-pigs. We made no human experiments, but have experimental data by others which has a direct bearing on this question.

Von Pirquet and Schick injected children with antitoxic horse serum at intervals.

It has been customary to immunize numbers of children when exposed to diphtheria with antitoxic horse serum at intervals of from three to four weeks.

We have no doubt that there are many such instances on record and, so far as we know, this practice has never caused death.

Repeated injections of horse serum into man is not an infrequent occurrence. Patients suffering with diphtheria are often given injections of antitoxic serum at short and frequent intervals. It is also not rare for persons to have several attacks of diphtheria at long intervals and to be treated each time with antidiphtheric serum.

Certain serums, for example the antitubercle serum of Maragliano, or the antirheumatic serum of Menzer, are habitually used by giving injections at intervals of days or weeks.

In all of these cases of frequent and repeated injections the amount which has been injected and the interval between the injections must be taken into account in relation to this work.

Von Pirquet and Schick in their work on Serumkrankheit give the following instances in which children received two injections of horse serum at intervals of sixteen to forty-two days between the first and second days:

Leopold H. — October 3, 1902. One hundred cubic centimeters scarlet fever serum (Moser). Eight days following this injection symptoms of the serum disease appeared and lasted several weeks.

December 2, 1902. Fifty days after the first injection, patient received two cubic centimeters antidiphtheric serum under skin of arm.

In fifteen minutes following this second injection stormy symptoms set in. The boy began to cry and complained of nausea. Edema of the lip set in and soon spread over the whole face. In several hours general urticaria.

Heinrich K. Three years old. — June 13, 1902. One hundred cubic centimeters scarlet fever serum. On the eighth day following the injection symptoms of the serum disease appeared which lasted until the twenty-sixth day.

July 7. Thirty-four days following the first injection, one cubic centimeter antidiphtheric serum injected into the left arm. The same afternoon urticaria and swelling of the lips. The next morning the arm was highly edematous.

Alexandrine K. Nine years old. — May 28, 1902. One hundred and eighty cubic centimeters normal horse serum. Eleven days after the injection severe serum symptoms appeared, which lasted until June 17.

Sixteen days following the first injection, given one cubic centimeter diphtheric antitoxic serum in the right forearm. Next morning, the hand swollen, very painful, and smartly reddened to such an extent that the infection was considered of an erysipelatous nature. All redness and swelling disappeared in a few days.

Elizabeth K. Six and one-half years old. — May 3, 1902. Given one hundred and eighty cubic centimeters scarlet fever serum. May 15, ten days following, sudden rise of temperature, 39.4° C. Swelling of the lymph nodes. No exanthem.

Nineteen days after the first injection fifty cubic centimeters scarlet fever serum, following which occurred severe and painful edema of the skin of the abdomen, which spread to the labia and thighs, and disappeared in about a week.

Franz Z. — June 6, 1903. One cubic centimeter antidiphtheric serum. June 16 had urticaria, about nine days after the injection. August 2, eight weeks after first injection, again given five cubic centimeters antidiphtheric serum. Shortly following, urticaria and swelling of the face.

Frieda Z. — June 10, 1903. One cubic centimeter antidiphtheric serum. June 26, sixteen days following, the same injection repeated and acted in all respects similar to the above case, Franz Z., her brother. On August 2 she was again given five cubic centimeters antidiphtheric serum. One hour later her face became red and swollen. The lids became so edematous that she could not open her right eye. In two hours there was general urticaria. Temperature 38.6° C. Moderate swelling, locally.

Elli M. Four months old. — First injection June 20, 1904. Twelve cubic centimeters antidiphtheric serum. Ten days later sudden rise of temperature 39° C. and urticaria. The fever lasted two days. Eruption later became measle-like and remained several days.

Nineteen days after the first injection five cubic centimeters antidiphtheric serum. One hour after this severe general urticaria. From the site of injection swelling of the skin of the abdomen. In the afternoon the temperature rose to 39.5° C.; frequent vomiting.

Leopoldine K. — December 1, 1903. Five cubic centimeters horse serum. Twenty-seven days following the first injection patient received one cubic centimeter antidiphtheric serum. One hour following this last injection definite swelling of the right hand, which later spread to the entire arm.

All these eight cases show in common, that after the first injection of horse serum the symptoms of the serum disease appear after the normal period of incubation, namely, between the eighth and thirteenth day. But when the same individuals are again injected with horse serum, after intervals of sixteen to forty-two days, there reappear at once or at least within twenty-four hours symptoms of the serum disease.

Von Pirquet and Schick further give a list of sixty children who were injected with antitoxic horse serum at intervals of six days to seven and one-half years between the first and second injections. They found that when the second injection was given from fourteen days to four months after the first injection they obtained, with great regularity, what they termed " the immediate reaction," but when the intervals between the first and second injection is over four months they obtain little or no immediate reaction, but what they termed "an accelerated reaction," for the fever, urticaria, and other symptoms of the serum disease appeared on the fifth, sixth, seventh or eighth day. It will be remembered that the normal period of incubation for the symptoms of the serum disease to appear after the first injection is between the eighth and thirteenth day. Von Pirquet and Schick lay special stress upon the phenomena of the " immediate" and "accelerated" reactions following the second injection.

We might also conclude that children may not be sensitized to the toxic action in horse serum by eating horse meat, from the fact that horse meat is a favorite article of diet in certain European countries, and there is nothing on record to show that the injection of horse serum in those countries is fraught with more danger than where this practice does not obtain. We must, however, remember that our work has shown that guinea-pigs are sensitized with exceedingly minute quantities of the strange proteid, and that repeated injections cause an immunity; and it is possible that the same action may be true of feeding.

Man reacts to the first injection of horse serum after a period of incubation of eight to thirteen days. Guinea-pigs show practically no reaction following the first injection. Both react to a second injection. The reactions in man and the guinea-pig, however, differ somewhat in severity and in kind. The relation, therefore, that our observations upon the guinea-pig may have in their application to man must await further study. Of course the fact that other animals besides man and guinea-pigs react to a second injection of horse serum would seem to indicate that we are dealing with one and the same action.

We have tested monkeys, rabbits, mice, dogs, cats, rats, chickens, and pigeons to determine whether any of these animals may be sensitized to the action of horse serum. Thus far we have obtained a response in the dogs, rabbits, and cats. This work is still in progress and will be reported at a future date.

SUMMARY AND CONCLUSIONS.

Normal horse serum, when injected into the peritoneal cavity of a normal guinea-pig, produces no symptoms. When injected subcutaneously there may result at most a slight local reaction, consisting of swelling and edema, which gradually disappears.

Antitoxic horse serum is equally harmless for normal guinea-pigs.

Horse serum is, however, poisonous to a guinea-pig which has previously been injected with horse serum. The "period of incubation" or time necessary to elapse between the first and second injection is about ten days. Under these circumstances, horse serum is poisonous whether injected subcutaneously or into the peritoneal cavity.

The first injection of horse serum renders the guinea-pig susceptible.

The symptoms caused by the injection of horse serum into a susceptible guinea-pig are respiratory embarrassment, paralysis, and convulsions, followed by death. The symptoms come on usually within ten minutes after the injection, and when death results it usually occurs within one hour, frequently in less than thirty minutes, and sometimes within a few minutes.

The poisonous principle in horse serum appears to act

FOOT-NOTE. — For the detailed account of our work, including the literature relating to it, see Bulletin 29, of the Hygienic Laboratory, U.S. Public Health and Marine Hospital Service, entitled "A study of the cause of sudden death following the injection of horse serum."

We have just received a paper by R. Otto entitled "Das Theobald Smithsche Phänomen der Serum-Ueberempfindlichkeit,"reprinted from Leuthold-Gedenkschrift, band I. Otto's paper deals with some of the problems we have studied, and his results are in harmony with many of our conclusions.

upon the respiratory centers. The heart continues to beat long after respiration ceases.

The toxic action of horse serum bears no relation to diphtheria. The poison is not toxone. Guinea-pigs cannot be rendered susceptible by previous infections with the B. diphtheriæ or by previous injections with diphtheria toxine.

It seems from our work, however, that guinea-pigs first injected with a mixture of diphtheria toxine plus horse serum are more sensitive to subsequent injections of horse serum than are guinea-pigs sensitized with a first injection of horse serum alone.

Diphtheria antitoxin plays no part in this poisonous action and in itself is harmless.

As soon as we realized that the toxic principle in horse serum exerts its action in quantities so minute as to place it almost in the category of the ferments, and, further, when we concluded from our work that this toxic principle is doubtless one of those highly organized and complex proteid substances belonging to the "haptin group" in the sense used by Ehrlich, we recognized how futile it would be with present methods to attempt to isolate this substance. Nevertheless, we devoted much time and study to the relation of this toxic principle to various chemical, physical, and electrical influences. The practical importance of eliminating or neutralizing this toxic principle in horse serum is at once evident.

It is probable that when the strange proteid is introduced into the guinea-pig it causes a reaction resulting in a production of "antibodies," so that when a second injection of horse serum is given there is probably either a union or a reaction between the antibodies and a substance in the horse serum which produces the poisonous effect.

This poisonous principle is quantitatively specific; that is, guinea-pigs treated with horse serum are rendered somewhat susceptible to the subsequent injection of the serum of another animal. Guinea-pigs treated with the serum of another animal are slightly sensitive to the toxic action of horse serum.

Guinea-pigs treated with the serums of various animals and subsequently injected, are much more susceptible to homologous serums than to heterologous serums.

This poisonous action has no relation to hemolysis. Our work proves that blood serum may contain an acute poison entirely independent of any hemolytic action. Normal horse serum has no lytic power upon the red corpuscles of the normal guinea-pig.

This poisonous action has no relation to the specific albuminous precipitins.

The poisonous principle in horse serum is not affected by a temperature of 60° C. for six hours, but it is destroyed at 100° C. for fifteen minutes.

The poisonous principle is filterable through porcelain, is not injured by drying, and cannot be separated by precipitation with ammonium sulphate and subsequent dialysis.

The following chemical substances do not oxidize, neutralize, or precipitate the poisonous principle in horse serum: Butyric acid, permanganate of potash, citrate of soda, alcohol, succinic peroxide acid (alphozone), hydrogen dioxide, and ammonium sulphate. The presence of chloroform or trikresol (0.4 per cent) does not interfere with this poisonous action.

Serums eight years old are as toxic as those freshly separated.

Exposure to X-rays does not affect the poisonous action of horse serum.

It requires about ten days after the first injection of horse serum for a guinea-pig to show susceptibility to a second injection. A guinea-pig remains susceptible a very long time, at least one hundred and sixty days.

As small a quantity as $\frac{1}{1000000}$ cubic centimeter of horse serum was sufficient in one instance to render a guinea-pig susceptible. Quantities varying from $\frac{1}{250}$ to $\frac{1}{1000}$ cubic centimeter almost invariably render guinea-pigs highly susceptible when given in the toxine-antitoxine mixture.

One-tenth cubic centimeter of horse serum injected into the peritoneum of a susceptible guinea-pig is sufficient to cause death. The same quantity inoculated subcutaneously may cause serious symptoms.

There is some evidence to show that the sensitizing substance in horse serum is the same as the poisonous substance. The sensitizing substance is not affected by precipitation with ammonium sulphate and dialysis.

Guinea-pigs may be sensitized with horse serum that has been dried and redissolved.

The sensitizing substance is not affected by a temperature of 60° C. for six hours.

It is probable that small quantities of horse serum render a guinea-pig more susceptible than do large quantities. If this be true, it is due, perhaps, to the fact that large quantities, owing to slow absorption or prolonged reaction, partly immunize the guinea-pig at the same time that it is being sensitized.

The sensitizing substance apparently is not free in the blood serum of guinea-pigs.

An active immunity against this toxic principle may readily be established by repeated injections of horse serum, at short intervals, into a guinea-pig. Although guinea-pigs may be immunized actively in this manner, we have not yet succeeded in transferring this immunity in the blood serum or body juices to another guinea-pig. It therefore appears that the immune bodies, if such exist against the toxic action of horse serum, are not free in the blood and body juices, contrary to the case in diphtheria.

Guinea-pigs may be sensitized to the toxic action of horse serum by feeding them with horse serum or horse meat.

The fact that guinea-pigs may be rendered susceptible by the feeding of strange proteid matter opens an interesting question as to whether sensitive guinea-pigs may also be poisoned by feeding with the same serum given after a proper interval of time. If man can be sensitized in a similar way by the eating of certain proteid substances, may not this throw light upon those interesting and obscure idiosyncrasies in which the eating of fish, sea food, and other articles of diet habitually cause sudden and sometimes serious symptoms?

The susceptibility to the toxic action of horse serum is transmitted hereditarily from the mother guinea-pig to her young.

These results upon the hereditary transmission of the susceptibility to the poisonous action of horse serum in guinea-pigs may throw light upon the well-known hereditary tendency to tuberculosis in children born of a tuberculous parent. There are certain analogies between the action of tuberculosis and horse serum. Both may produce a hypersusceptibility and also a certain degree of immunity. Now that we have proved that this hypersusceptibility or anaphylactic action in the case of horse serum may be transmitted hereditarily in guinea-pigs, may it not throw light upon the fact that tuberculosis "runs in families"?

Demonstrations of the hereditary transmission of acquired characters are comparatively rare in biology. While there are several recorded instances demonstrating that immunity to certain infectious diseases may be transmitted from a mother to her young, yet, as far as we know, this is the first recorded instance in which hypersensitiveness, or anaphylaxis, has been experimentally shown to be transmitted from a mother to her young.

Other albuminous substances, such as skimmed milk, peptone, hemoglobin, egg albumin, and vegetable proteids, possess no poisonous action upon guinea-pigs sensitized with horse serum. Whether guinea-pigs are rendered susceptible to a subsequent injection with the same albuminous matter with which they have been sensitized will be reported in a later paper.

We believe that the substance which sensitizes the animal is identical with that which later poisons it. However, the substance must first cause a reaction in the organism resulting in a production of antibodies. We have found that small quantities of horse serum produce, after a definite period of incubation, a condition of anaphylaxis; multiple or repeated injections produce immunity. We therefore possess in horse serum a substance capable of causing both anaphylaxis and prophylaxis.

It may be that man cannot be sensitized in the same way that we have shown to be the case with guinea-pigs. Children have, in a number of instances, been injected with antidiphtheric horse serum at short and long intervals without, so far as we are aware, causing death. Certain serums, for example the antitubercle serum of Maragliano and the antirheumatic serum of Menzer, are habitually used by giving injections at intervals of days and weeks. In all such cases of frequent and repeated injections the amount which has been injected and the interval between the injections must be taken into account in relation to our work. Von Pirquet and Schick have shown that a second injection of horse serum into children causes an "immediate" or an "accelerated" reaction. Both the immediate and the accelerated reaction in children are characterized by symptoms of "the serum disease."

We might conclude that children may not be sensitized to the toxic action of horse serum by eating horse meat, for horse meat is a favorite article of diet in certain European countries and there is nothing upon record to show that the injection of horse serum in those countries is fraught with more danger than where this diet is not used. It should, however, be borne in mind that our work has shown that guinea-pigs may be sensitized with exceedingly minute quantities of a strange proteid, and that repeated injections cause an immunity, and it does not seem impossible that the same action may be true of food.

Man reacts to the first injection of horse serum after a period of eight to thirteen days; guinea-pigs show no reaction as a result of the first injection; both man and guinea-pigs react to a second injection. The reactions in man and the guinea-pig differ, however, both in severity and in kind. The relation, therefore, that our observations upon the guinea-pig may have in their application to man must await further study.

The fact that other animals beside man and the guinea-pig react to a second injection of horse serum would seem to indicate that we are dealing with one and the same action.

We believe that our results make it probable that man may be rendered sensitive to the injection of a strange proteid, as is the case with the guinea-pig and other animals, and that this explanation must be considered as well as the *status lymphaticus*, which has heretofore been assigned as the cause of sudden death following the injection of horse serum.

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A STUDY OF THE SO-CALLED INFECTIOUS LYMPHOSARCOMA OF DOGS. *

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For many years the attention of pathologists has been attracted by a venereal disease of dogs, characterized by transmission through coitus, by the presence of nodular tumors of the genitals, multiple secondary growths of the skin, metastases in the inguinal and abdominal lymph nodes, accompanied by cachexia, and often terminating fatally.

The most significant feature of the disease has been the marked infectiousness of the process, both by natural contagion and by experimental inoculation, but the important bearing of the malady on the general problem of tumors and the unusual facilities which it offers for the experimental study of a bulky tumor readily transmissible between comparatively large animals seem not to have been fully recognized until recently.

Clinical features. — The disease is evidently of general distribution, having been described in Russia, France, Germany, and England, and we have had no difficulty in securing numerous cases in New York City.†

Young or middle-aged adult dogs of both sexes are chiefly affected. Instances of the spontaneous transference of the disease *per coitum* are reported in detail by Smith and Washbourn, and Powell White, and are commonly observed by veterinary surgeons. In the female the disease begins with small nodular or papillary growths, single or multiple, of the vaginal mucosa, whence it extends by metastasis to the neighboring skin, pelvic, and abdominal lymph nodes, and occasionally to more distant organs. In the male the corona glandis is usually first involved by a nodular infiltration with secondary cutaneous tumors, sometimes involving the entire penile sheath. A catarrhal inflammation usually precedes the development or discovery of the tumor, but in many cases the growth appears without any inflammatory irritation. In both sexes isolated growths of the skin may occur in the groin, abdomen, back, neck, and hind or fore legs, as well as in the abdominal lymph nodes, mesentery, spleen, liver, kidney, and lung.

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† Our material has been secured entirely through the efforts of Dr. T. G. Sherwood, of New York, for whose energetic coöperation we take pleasure in expressing our sincere thanks.

The superficial tumors may ulcerate or necrose, but the protected growths are usually found intact. The growths are usually well circumscribed, encapsulated, and free from any signs of inflammatory reaction, but in older cases there may be diffuse infiltration of the skin or mucous membrane which, becoming eroded or ulcerated, may be considerably inflamed and yield a serous or purulent discharge.

In the larger tumors considerable areas may necrose and be sloughed out. Spontaneous regression may occur through a process of fibrosis and atrophy, and permanent disappearance of the disease in this manner has been noted. Local treatment has considerable influence in checking the progress of the disease if instituted early, and Dr. Sherwood informs us that by repeated curettage and application of caustics the early stages of the genital lesions may usually be arrested.

Clinical conditions in the dog resembling infectious lymphosarcoma. — Sticker describes two infectious inflammatory diseases of dogs which resemble the infectious lymphosarcoma. (1) The pointed condyloma of the genitals arises after long continued irritation and produces small papillary excrescences which often reach a considerable size and may produce large firm lobulated outgrowths. Histologically they consist of hypertrophic epithelium supported by new vascular connective tissue infiltrated with round cells. (2) An infectious follicular vaginitis or balanitis arises in the course of chronic purulent catarrh and produces multiple tumors of the size of a pea or bean, composed of enlarged lymph nodules which displace the epithelial lining outward.

We have encountered in Dr. Sherwood's clinic cases apparently belonging to the class of follicular vaginitis, but we do not feel certain that such cases can be satisfactorily distinguished from early lymphosarcoma. According to Sticker the true tumor is usually single and may be unaccompanied by catarrhal inflammation, and its cells are of large size, while follicular vaginitis gives multiple superficial tumors, is always marked by purulent discharge, and the lymphoid cells are smaller. Yet Smith and Washbourn state that a constant leucorrhea is the usual sign of the presence of this tumor, and the multiple tumors which we have examined in cases of vaginitis have shown much the same structure as the metastatic lymphosarcoma. We are inclined, therefore, to suspect that there is a close connection between Sticker's follicular vaginitis and the true lymphosarcoma which many authors have been studying.

Dr. Sherwood assures us that all cases of vaginitis in dogs with submucous nodular growths, if untreated, go on to develop the characteristic lesions of lymphosarcoma, and that the only other form of chronic vaginitis in dogs, as in the human subject, produces only small granular elevations of the epithelium, but never any definite tumors of the size of a pea or bean. We are, therefore, not ready to accept Sticker's description of an infectious inflammatory hyperplasia of the submucous lymph nodules as a separate disease from the lymphosarcoma of various authors.

(3) In one case, which we have recently observed with Dr. Sherwood, an old dog presented much more extensive lesions of the type of lymphosarcoma than any we have found described. The skin of the genitals and groin was thickly infiltrated with many flat masses over which the skin

was eroded, dry, and shiny. There were several large tumors and many smaller ones on the back, and hind and fore legs. The skin of the lips, face, and head was beset by a large number of nodules or flat plates covered by reddened shiny cuticle. The lymph nodes of the groin, pelvis, abdomen, and those about the jaws and neck were much enlarged by extensions of the tumor process. The spleen was nearly doubled in size by many small nodules which had the distribution of the Malpighian bodies. On section of this organ, the pulp was found to contain many islands of nucleated red blood cells interspersed with many typical myeloid giant cells. In the liver the portal canals were extensively infiltrated by masses of small round cells. The structure of all these tumors was that of small cell lymphosarcoma. On account of the small size of the cells lying in a lymphoid reticulum, the myeloid changes in the spleen, the universal distribution of the tumors, and absence of leucocytosis, we regard this case as one of lymphadenia and quite different from the infectious lymphosarcoma. We have encountered one other case of this type in which the liver was chiefly involved. The extensive involvement of the genitals might readily lead to the error of mistaking this disease for the infectious lymphosarcoma.

The above observations, and other peculiarities briefly noted in the literature, emphasize the need of more thorough clinical study of the infectious tumor than it has yet received. Several important problems seem to await solution largely through such clinical study. It is possible that more than one disease has been included in the studies of various observers, and that some conflicting results may therein find an explanation. More minute investigation of the beginnings of the disease may throw important light on the etiological factors. The fully developed spontaneous disease ought to display more clearly the true position of the malady among pathological processes. The relations of the entire group of lymphosarcomata are here brought into question under conditions unusually favorable for their experimental study.

Gross and microscopical appearance of the spontaneous tumors. — The tumors are rather soft, elastic, well-circumscribed masses of grayish white tissue yielding a mucinous fluid on pressure, or they may be firmer and contain more fibrous tissue, especially when regressing. In size they vary from early minute nodules to multiple growths as large as an orange. Caseation is not observed, but large areas in voluminous tumors may necrose. Microscopically, the cells are uniformly large, round or polygonal with pale staining or clear protoplasm and single nuclei with prominent nucleoli and often showing mitotic figures. They much resemble the large proliferating cells of lymph follicles. These cells are arranged not in alveoli, but in cords supported by thin strands of connective tissue, or, in rapidly growing specimens, there are diffuse areas of cells, while in regressing cases the connective tissue may appear in numerous isolated masses of considerable bulk. The blood vessels are moderately developed. Multiplication of cells occurs in all parts of the tumor, producing a growth which displaces the surrounding connective tissue outward as a capsule, which is entirely free from signs of inflammatory reaction.

Our studies have not furnished satisfactory grounds for the exact histological classification of this tumor. While it most resembles a large cell lymphosarcoma, the cells are polygonal more often than round, the fine reticulum of a lymphosarcoma is missing, the growth is much less malignant than large cell lymphosarcoma in man, the arrangement of the cells in alveoli without lumina is sometimes distinct, the protoplasm of the cells is nearly homogeneous, and a mucinous discharge from the cut surface of the tumors is characteristic. The general histological characters suggest to us the diagnosis of alveolar sarcoma or endothelioma. The particular tissue cells giving origin to the tumor have never been determined.

Experimental inoculation. — Nowinsky (1877), who first described the disease in detail, also reported its successful transference by inoculation of the tumors. From an ulcerated recurrent tumor of one year's growth in the vagina he transplanted pieces to the subcutaneous tissue of three young dogs, and in the course of three or four months saw the development of isolated tumors as large as a walnut or egg. The structure of all the tumors was that of myxosarcoma. No further study of the condition appears to have been made until 1888–9 when Wehr reported the successful transfer of several of the tumors in a series of twenty-four dogs. In one of these, after a year's growth, there were found numerous metastases in the peritoneum and abdominal organs. Since that time the tumor has been studied and numerous successful inoculations practised, by Duplay and Cazin, Geissler, Smith and Washbourn, Powell White, Sanfelice, Sticker, and Bashford, Murray, and Cramer.

The observations of these authors have demonstrated many important facts regarding the inoculability of the process. The animals successfully inoculated have included many breeds, all varieties of dogs appearing to be about equally susceptible. Sticker transferred the tumor from a dog to two foxes, but failed to secure growths in cats, guinea-pigs, rabbits, rats, and mice. Young animals have been chiefly employed, but Smith and Washbourn found that the tumors grew larger in older dogs, and that a moderate proportion of animals possess complete natural immunity.

The course of successful implantations is usually quite uniform. When inserted beneath the skin a period of two or three weeks elapses before any definite enlargement is noted. Thereafter the nodule may steadily increase in size for four or five months, producing a well-circumscribed tumor as large as a hen's egg or small orange. Smaller secondary tumors may develop in the neighborhood, or in the abdomen, spleen, liver, and lung. In progressive cases the tumors reach their largest dimensions in three to six months and the animals die with cachexia and emaciation, often aggravated by sloughing and infection of the larger masses. At any period the tumors may cease to grow and a regressive process begins which ends in a complete atrophy, after which the animals have usually been found completely immune to reinoculation. Sticker reports spontaneous disappearance of subcutaneous tumors in eighteen out of sixty experimental cases. It has been remarked by Sticker and has been our experience, that while the spontaneous disease often extends through the lymph nodes, the subcutaneous experimental tumors are usually fatal, without affecting the lymph nodes. Geissler, however, saw extensive involvement of peritoneum, abdominal nodes, and spleen, eight months after inoculation of the ventral skin. Sticker observed six cases in which tumors implanted in various localities directly invaded lymph nodes, a course which he referred chiefly to the influence of complicating infections of the tumor. Besides the regional metastases Sticker reports several cases of general sarcomatosis following inoculation, with numerous nodules in the skin, spleen, liver, kidneys, and heart and skeletal muscles, pointing to dissemination through the blood vessels.

Intraperitoneal inoculation was extensively practised by Sticker. Here small multiple tumors of the omentum developed in two months, and in three months diffuse sarcomatosis throughout the peritoneum. Some of his omental tumors reached the size of a cocoanut. Extensive growths followed inoculation of the pleura and tunica vaginalis, while successful implantations were made in the stomach wall, liver, lung, orbit, and bone marrow.

It has been a remarkable feature of the inoculation experiments that by merely rubbing the scarified mucosa with portions of the tumor successful results have been secured. Smith and Washbourn, Duplay and Cazin, and Sticker, all report that after such procedure the inflammatory reaction subsides and in a few weeks single or multiple tumors develop. Complete comminution of the tumor cells destroys the inoculability of the material and filtrates of tumor emulsions passed through paper or earthen filters are innocuous. The tumor cells are still viable after exposure for twenty-four hours at — 14° C., but are killed when heated for two hours at 54° C. (Sticker).

The implantation experiments which form the basis for this report were begun in the spring of 1905, and the first plants were made from a tumor of the vagina removed at operation by Dr. Sherwood. A section of the growth used for implantation has in every case been kept for histological examination. These earlier plants were made by two methods, viz.: implantation subcutaneously of a thin section of tumor tissue, and infusion of a suspension of the tumor cells into one of the mesenteric veins.

The tumor pulp was strained through gauze before inoculating. Under these conditions the meshes of the gauze become filled with the gelatinous fragments of tumor tissue, so that only the very finest particles can pass. Such filtrates give much fewer successful inoculations than are obtained when larger particles are included.

The material was brought to the laboratory from the operating room in a special double walled vessel so arranged that a water jacket kept the inner chamber at body temperature. In view of the extraordinary vitality of the tumor cells such a precaution was probably unnecessary. These four earlier experiments made with growths removed from the vagina gave negative results. Previous to the operative removal, one of the tumors from which plants were made had been subjected to treatment by curettage and the application of caustics. It is quite probable that this procedure had interfered with the normal vitality of the cells.

For the later experiments, begun in the fall of 1905, the tumor tissue was obtained from the left inguinal lymph nodes of a five-year-old male Irish terrier. The primary growth was found on the sheath of the penis, but the penis itself was not involved in this case.

A number of dogs were planted with small grafts subcutaneously on the abdomen, others by subcutaneous injection of a suspension of the tumor cells. All of the animals which had been planted by the first method developed tumors in from three to five weeks. The second group were failures. All of the tumors which we have studied experimentally have come from this successful implantation.

The tumor is now in its fifth generation and continues to grow with undiminished vigor. One hundred and twenty-two plants by various methods have been made on twenty-nine dogs of various ages and breeds. From this number thirty-five tumors have developed and there have been twelve spontaneous recoveries.

Certain generalizations may be drawn from the statistics and conditions of these experiments: Old dogs grow the tumor better than young animals. The rate of growth is in a rough way dependent upon the general health of the animal. With poor nutritive conditions the growth is rapid and improvement of health following operative removal of a portion of the tumors is often accompanied by a regression of the remainder. Spontaneous recovery is a common phenomenon, and generally occurs in young, vigorous animals. Four recovered animals we have been unable to infect a second time. In a susceptible animal only a portion of the plants may grow. If one tumor starts to regress, all do. We have never seen some tumors regressing and others growing vigorously in the same animal at the same time.

The crucial point which we have attempted to settle in this study has been the question of the mode of origin of the tumor, *i.e.*, whether the growth comes from the proliferation of the transplanted cells or by stimulation of the surrounding tissue cells, as maintained by Bashford, Murray, and Cramer. The small sections which we have planted for this purpose have been taken from actively-growing tumors and have been planted in other locations on the same animal that furnished the seed. By such procedure we are sure of having a susceptible animal, but in such conditions not every plant will grow.

The small operation wounds healed kindly, and after waiting the desired interval, the plant, together with the surrounding tissue, was removed and preserved for histological study.

It may be a point of considerable importance to note that the operation wounds, from which we afterward removed tumor grafts, healed in every case by primary union, so that the inflammatory reaction was caused only by the traumatism of the operation. It is readily understood that the particular changes which we wished to observe might easily be obscured by the inflammation following infection.

Histology of the transplanted tumors. — Throughout our entire series of transplantations the structure of the tumor has remained remarkably uniform, agreeing in this respect with the experience of all previous observers. The cells always maintain their large size, round or polygonal form, single nuclei with prominent nucleoli, clear or slightly granular protoplasm with indifferent affinity for eosin (Muller-formol fixation), and arrangement in cords without alveolar lumina, supported by thin strands of connective tissue. Sharp demarcation from the surrounding tissue and absence of round cell infiltration and caseation, are nearly constant features of uncomplicated growths. When the tumors regress there is a focal thickening of the trabecula with simple or degenerative atrophy of the cells, without necrosis. The surrounding tissue is displaced mechanically by the tumor and is usually quite free from signs of inflammation or participation in the growths.

Histological study of the fate of the transplanted cells. — For this purpose pieces of the tumor, implanted in the manner described, were excised with the surrounding tissue at intervals of one to twenty-one days, hardened in Muller-formol, cut in paraffin, and stained by hematoxylin and eosin.

Twenty-four-hour specimens.— Pieces excised after twenty-four hours are found to be lying loosely adherent to the surrounding tissue. On section the nodules are lying in a mass of fibrin, serum, and red blood cells, while polynuclear leucocytes are abundant in the surrounding vessels and blood clot. The compact nodule of tumor tissue is entirely necrotic except for a few islands of ten to fifty intact cells, lying along the periphery of the piece. These islands are clearly shown to belong to the transplanted piece by their morphology, immediate relations to the necrotic central portions, and separation from the host's tissues by a well-defined layer of fibrin or leucocytes. Not all the cells in these islands are intact. Some are necrosed, others degenerating, most are normal, and a few show mitotic figures (Plate XIX., Figs. 5, 6).

Forty-eight-hour specimens. — After two days the nodules are more firmly adherent, and the only histological change noted is increased edema and diminished leucocytosis in the surrounding tissues of the host. The peripheral islands of tumor cells appear as before and it now becomes evident that these islands are in contact with small blood vessels, apparently those of the original tumor, which contain normal red blood cells as though circulation had become restored in them. The sharp separation of most of the islands from the host's tissues is even more apparent than before (Plate XVIII., Fig. 4).

Three-day specimens. -- At this time the implanted piece has become well fused with the subcutaneous tissue of the host, which remains edematous and infiltrated with polynuclear leucocytes. The peripheral islands of intact cells are now usually fused into a definite layer surrounding the necrotic center and are clearly visible to the naked eye in the stained section. In this layer are many blood vessels containing blood which appears to have been in physiological condition. Some of these vessels must have belonged to the original tumor since they are too large to have been developed in three days. The outer edges of the layer of tumor cells are again sharply separated by strands of fibrin from the host's tissues, and we could find no trace of the transformation of fibroblasts or any other cells of the host into tumor cells. At this stage many of the tumor cells show mitosis (Plate XIX., Fig. 7).

Six-day specimens. — The peripheral layer of tumor cells has now increased in depth, while the necrotic central mass has undergone some absorption, and edema and leucocytosis have largely disappeared. The tumor cells have in some segments displaced the host's tissues before them, forming a thin capsule which separates the tumor from the subcutaneous fat and connective tissue. At other points this separation is less complete and here the tumor cells seem to be mingling with the multiplying fibroblasts of the host. Even in such foci, which are rare, the tumor cells are usually distinguishable from the fibroblasts, and we are unable to see any definite evidence of transformation of fibroblasts into tumor cells. Such areas, however, have doubtless formed the basis of the claim by Bashford, Murray, and Cramer, that the tumor develops exclusively from infected subcutaneous fibroblasts which become transformed into tumor cells. Such areas of mingled fibroblasts and tumor cells are rare in our specimens. All but small portions of the circumference of our implanted nodules are, from the first, sharply separated from the surrounding connective tissues by fibrin, fat, or acellular connective tissue, and in by far the greater part of the growing mass we find it impossible to conceive how the growing cells can be derived from the host's cells (Plate XIX., Fig. 8).

Fifteen days. — At this time the nodule has reached the size of a pea, the necrotic material has been absorbed, the cells are actively multiplying by mitosis. Their arrangement is identical with that of other tumors, and the entire mass is sharply separated from the surrounding tissue by a definite capsule. The surrounding tissue is rather vascular, but exhibits no other signs of inflammation (Plate XX., Fig. 9).

At twenty-one days the appearances have been exactly similar to those found at fifteen days.

Biological studies. — Sanfelice has been the only observer to report positive results from the search for microörganisms in the tumor. He observed in fresh preparations of fragments removed from a vaginal growth refractile double contoured bodies which he regarded as yeasts. These he was able to cultivate on ordinary media, and the culture proved pathogenic for small animals, but caused only inflammatory reactions. Sticker conducted systematic cultures from the growths on various media under aërobic and anaërobic conditions. The results were negative. Smith and Washbourn, Bashford, Murray, and Cramer, and Sticker, all mention the comparative freedom of the cells from the usual forms of cell inclusions.

On account of the clinical resemblance of the disease to syphilis, we have regularly examined material from the original growths and from the experimental tumors for spirochetæ. Spreads were made from the affected mucous surfaces and from the interior of tumor masses, and stained by Procas or Goldhorn's methods. From some cases the tissue was prepared according to Levaditi's second method for the demonstration of spirochetæ. In seven clinical cases we failed to find any spiral organisms, which seem not to be common saprophytes of the genital organs of dogs. In one large multiple tumor, growing from a transplanted piece, which was sloughing extensively, some large spiral organisms, resembling Spirocheta refringens, were found on the necrotic surface and within well-preserved portions of the tumor. In all other cases the results were negative. Spreads from actively-growing tumors are remarkably free from bodies resembling microörganisms, and from the usual cell inclusions of tumors.

NATURE OF THE INFECTIOUS LYMPHOSARCOMA OF DOGS.—The foregoing description of the clinical and pathological characters may form a basis for discussion of the nature of the disease. If the process is an infectious granuloma it is of some practical interest to dog fanciers, and from the standpoint of descriptive comparative pathology, but if it is a true neoplasm, it possesses very great practical and theoretical importance for the general problem of malignant tumors.

From the beginning of the observations on the disease up to the present time opinions regarding the nature of the process have been at variance. Nowinsky and Wehr speak of the growth as a carcinoma, but do not appear to have attempted a careful distinction between carcinoma and sarcoma. Geissler at first regarded his tumor as a carcinoma on account of the alveolar arrangement (he did not speak of lumina in the alveoli), but the discussion of his communication favored the diagnosis of sarcoma. The description indicates that his specimens were infiltrated with leucocytes. Hansemann, from a cursory examination, pronounced the growths exhibited by Geissler to be granulation tissue or sarcoma. Duplay and Cazin were uncertain as to the nature of their tumor. Smith and Washbourn, Sanfelice, White, and Sticker are certain that their tumors must be regarded histologically as round cell sarcoma. Bashford, Murray, and Cramer believe that they have proven that the process is an infectious granuloma, finding that the transplanted cells necrose while the new tumor grows from the infected cells of the host.

Sticker secured specimens of the tumors studied by Wehr, Geissler, Smith and Washbourn, Sanfelice, and Bashford, and submitted them, with his own, to a large number of German pathologists, all of whom agreed upon the diagnosis of round cell sarcoma.

Supported by this diagnosis of recognized authorities on the structure of tumors, Sticker compares the general features of the disease with malignant tumors in man and shows that on all these grounds the growth must be accepted as a true malignant neoplasm.

(1.) Clinically the other infectious venereal diseases of dogs resembling lymphosarcoma always cause prolonged inflammation and multiple nodules, which are merely enlarged lymph follicles, while inoculation of the vagina by the true tumor produces little or no inflammation, which soon subsides and is followed by solitary, or at best two or three, tumors arising in the subcutaneous tissue.

(2.) Histologically, the tumor is entirely different from any known infectious granuloma, is not accompanied by any inflammatory reaction in the neighboring tissues, and produces metastases which develop from cells carried by the blood stream, and without participation of the tissue cells.

(3.) Experimentally it is necessary to transfer living cells in order to secure a growth, while tumor emulsion finely comminuted, or strained through paper or porcelain is innocuous.

(4.) The spontaneous transference of the tumor is paralleled by the cases of " cancer á deux," and of epithelioma of the upper transferred from the lower lip in man.

(5.) The spontaneous disappearance of the tumor in dogs is quite comparable to the occasional disappearance of sarcoma and even of carcinoma (Orth) in man.

It must be admitted that Sticker's argument presents a body of evidence which speaks very strongly in favor of the opinion that the infectious lymphosarcoma of dogs is a true tumor. Our experience with the disease fully supports his views, with the possible exception that we are unable as yet to satisfactorily distinguish between his follicular vaginitis and the lymphosarcoma.

Yet, in spite of the opinions of many eminent pathologists based on the histology of the growths, the whole argument appears fatally defective when direct experimental evidence, as furnished by Bashford, Murray, and Cramer, indicates that the transplanted cells die and the tumor grows by infection of other tissue cells. This is the crucial test of an infectious granuloma as against a neoplasm, and if the observations of the English Commission are correct the decision, in our opinion, lies conclusively in their favor. In any event, Bashford, Murray, and Cramer have discerned the true method for determining the nature of the process and the futility of relying upon the histological appearance of the tumors, no matter how experienced the authorities consulted.

Our study of the disease was undertaken with the same object of determining the fate of the transplanted cells and, in spite of preconceived opinions, we had already reached a contrary conclusion when the results of the English Commission came to hand.

As indicated in the description of the changes occurring in the transplanted pieces of tumor, we are unable to confirm the results obtained by Bashford, Murray, and Cramer. In our specimens all the cells of the transplanted pieces do not perish, but a few peripheral islands of cells always remain alive, showing mitotic division from the first day, and separated from the tissues of the host so clearly as to leave no doubt that the transplanted cells give rise to the tumor. Between the third and fifth days, when, according to the English Commission, all the transplanted cells have perished, our specimens show very active mitosis in a definite layer of tumor cells separated from the surrounding tissue by fibrin. In our experiments the critical time for the transplanted cells comes in the first forty-eight hours when most of the cells necrose, after which mitotic figures in the remaining cells become very numerous.

That some tissue cells may be included in the tumor or be transformed into tumor cells we are not prepared to deny, although there is no definite evidence of such a process in our specimens.

We are unable to explain fully these contrary results of observations on the fate of the cells. It seems possible that Bashford and his associates were dealing with a less active tumor than ours, and with more resistant animals, and that the surviving cells in their experiments were too few to be recognizable as belonging to the transplanted nodule. This suggestion appears the more plausible since these observers state that the transformation of tissue cells into tumor cells is noted chiefly about slowly-growing tumors which are not well encapsulated. Such slowly-growing tumors, however, are less suitable for revealing the true capacities of the cells for independent existence. They admit, also, that in more rapidly-growing tumors the chief part of the growth arises from the tumor cells, while the transformation of fibroblasts is not always in evidence. Yet in progressive infectious granulomata, tissue cells are always being involved in the lesion, and the recorded observations of Bashford, Murray, and Cramer do not seem to fully support their conclusions regarding the nature of the process.

It may be suggested, also, that if an infected tumor were used for transplantation, the inflammation resulting from the bacterial infection might obscure the fate of the transplanted cells.

In view of all the clinical and pathological characters of the disease described by previous observers, many of which we have been able to verify, and of the results of the attempt to trace the fate of the transplanted cells, we are forced to conclude that the infectious lymphosarcoma of dogs is a true malignant neoplasm, of which the cells show, perhaps, a greater capacity for independent existence and infectivity than is seen in any other known tumor process.

The highly infectious nature of the process led Smith and Washbourn to regard the instance of this disease as a powerful argument in favor of the parasitic origin of malignant tumors. If, however, the transplanted cells continue to grow and produce the tumor, then the infectiousness of the process becomes an equally strong argument against a parasitic origin.

Yet the experimental evidence will not permit us to decide positively for or against the parasitic theory. There is the possibility that the tumor cell may contain within itself some form of microörganism which is the source of the peculiar form of stimulation that is the essential factor in the growth activities of the host. We can, however, formulate certain conditions which the hypothetical parasite must follow in this particular instance without committing ourselves to the declaration that such conditions are universal for tumor growth.

We have no knowledge of the parasite outside of the host cell. All those means of comminution which destroy the integrity of the tumor cell stop at the same time its infectiousness. The parasite, if it does exist outside of the host cell, is harmless. Those forms of injury which affect the tumor cell injure at the same time and to the same degree the intracellular parasite. This parallel is particularly interesting with reference to cold. Sticker has found it possible to cool the cells to — 14° C. and still have growth. Now — 14° C. is that critical temperature which Folin has found for the protoplasm of frog's muscle. If exposed from — 15° to — 21° C. the muscle goes into rigor on warming, while at —14° C. or above, the tissue is still irritable when thawed.

The critical temperatures of heat and cold of host cell and parasite must be identical. The very careful biological study of the growth indicates that the parasite must be of ultramicroscopic size, or that its protoplasm has such close agreement in staining properties with the tumor cell, and has such an homogeneous arrangement within the latter as to be impossible of discernment by methods now at our disposal. Our only knowledge of the parasite's existence is its supposed responsibility for the growth activities of the tumor. Since the life of the parasite and the manifestation of its activities in producing a malignant growth are absolutely dependent upon the transfer of a living tumor cell from one individual to another, there can be no objection to considering the tumor cell the essential infective agent in the process of transmission. Such conditions imply a symbiotic relation between the two consociating cells that is without analogue in such cases as have already been investigated either in the animal or the vegetable kingdom. The conditions require so intimate and vital a relation between parasite and tumor cell that it is impossible to make any distinction between them.

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DESCRIPTION OF PLATES.

PLATE XVIII. — Fig. 1. Section of original tumor used for transplantation. x 200.

Fig. 2. First day after transplantation. x 320. Island of living cells in edge of transplanted piece. Below is a layer of leucocytes and fibrin.

Fig. 3. First day after transplantation. x 200. Layer of living cells adjoining layer of fibrin and leucocytes, to right, and necrotic tumor, to left.

Fig. 4. Second day after transplantation. x 200. Thin layer of living cells on edge of necrotic tumor, and adjoining fat and fibrous tissue.

PLATE XIX. — Fig. 5. Mitotic nuclei in living cell layer of Fig. 4. x 600.

Fig. 6. Second day. Layer of living cells. x 330.

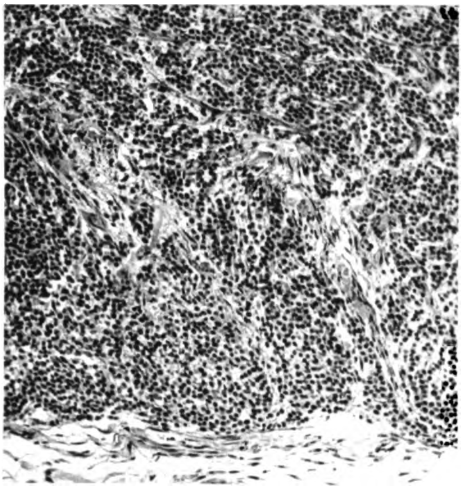
Fig. 7. Third day. Deeper layer of living cells grading into necrotic tumor, to left, and sharply separated by band of fibrin from host's tissues, to right. x 330.

Fig. 8. Sixth day. Deep layer of growing cells adjoining necrotic tumor above, and edematous tissues of host below. x 200.

PLATE XX. — Fig. 9. Fifteen days. Sharply circumscribed tumor. Compare original piece, Fig. 1. x 330.

Fig. 10. Appearance of whole transplanted piece after three days. On the right the necrotic center is surrounded by a lighter layer of proliferating tumor cells A dark layer of fibrin surrounds most of the circumference.

Fig. 11. Tumor in situ.



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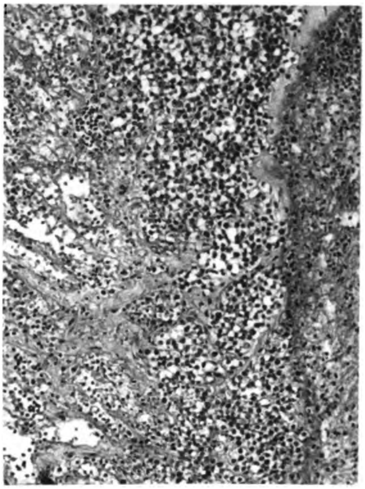

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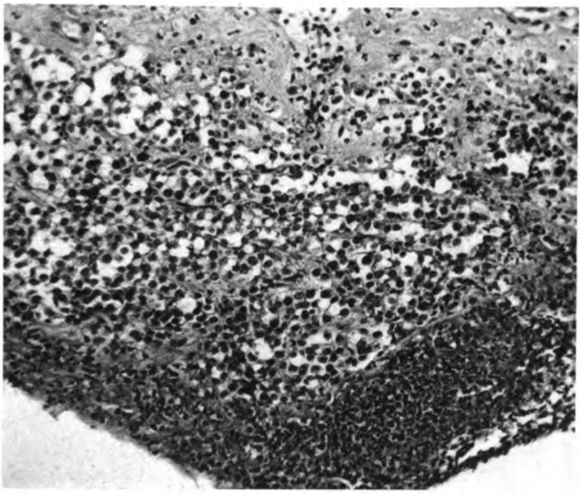

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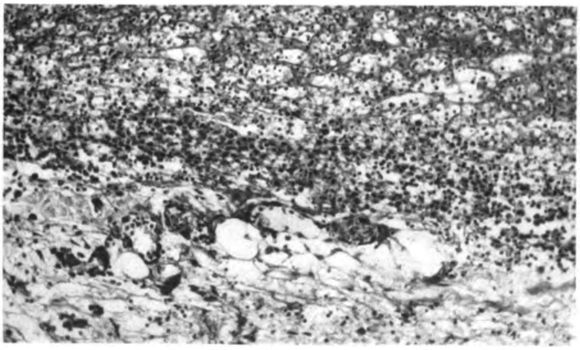

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Beebe and Ewing.

Lymphosarcoma of dogs.

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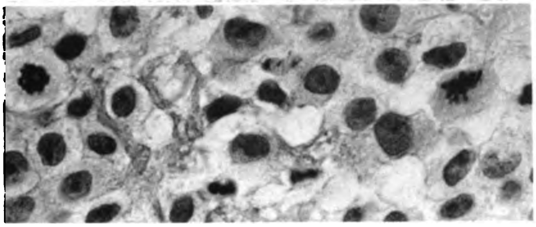

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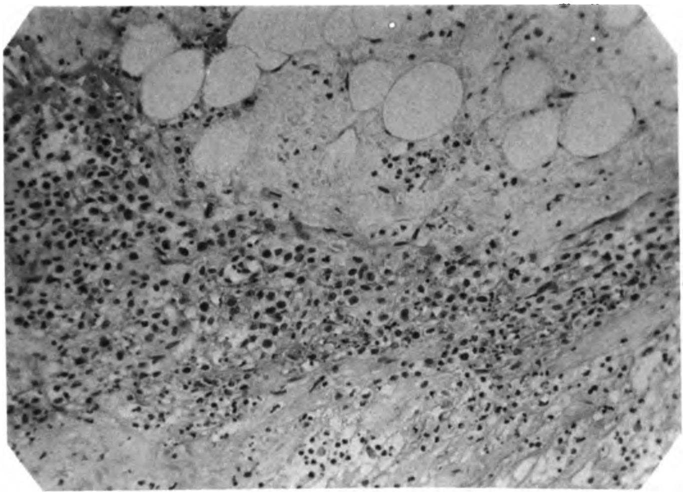

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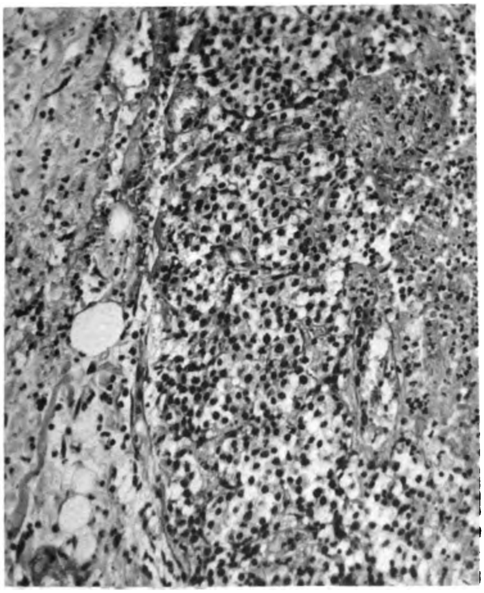

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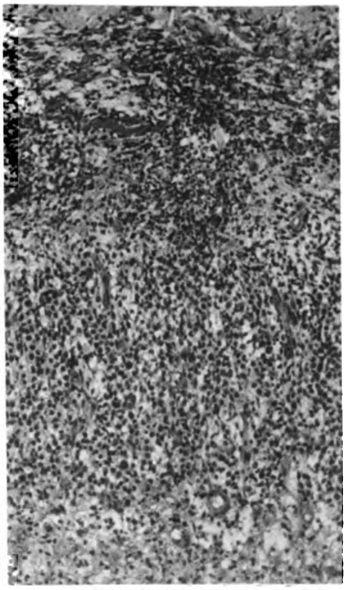

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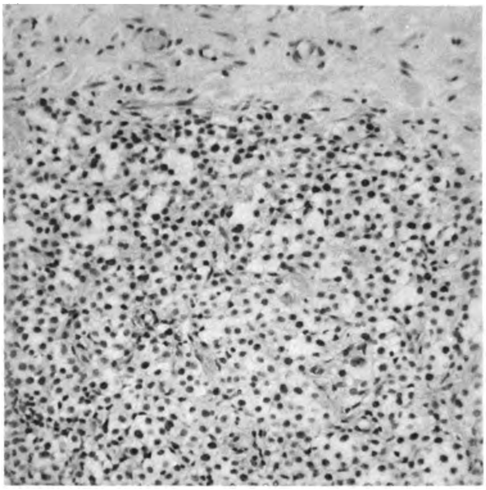

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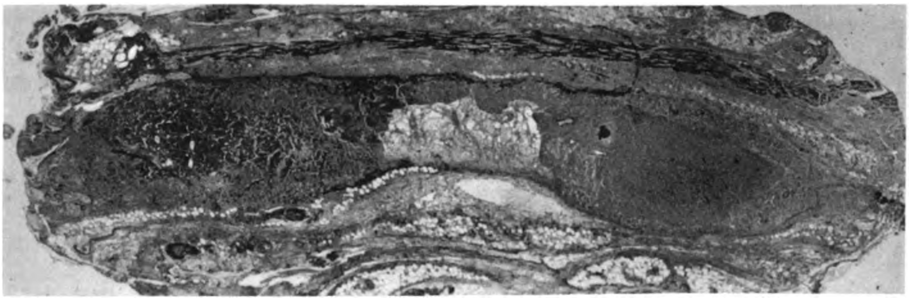

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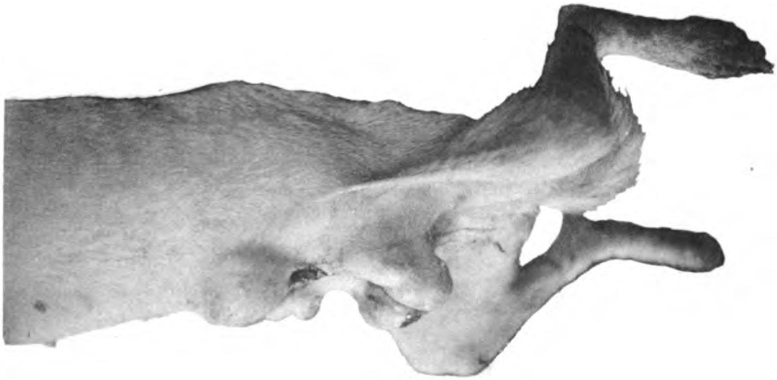

11

INTRAVENOUS INJECTIONS OF NICOTINE AND THEIR EFFECTS UPON THE AORTA OF RABBITS.*

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Several years ago, one of us[1] reported a series of experiments undertaken with a view of testing the effect of chronic tobacco poisoning upon the arteries of rabbits. The tobacco was given by mouth in the form of an infusion in varying strength and quantity and for a long time. As a result of these experiments, some very interesting lesions were found in the liver, but the arterial system showed no pathological changes whatsoever.

The results obtained by Josué, Erb, and others suggested to us the investigation of the effects of nicotine applied intravenously. When we commenced our experiments, about a year ago, we were fortunately not aware of the fact that Josué[2] had experimented with nicotine injections and had obtained no results. It may be stated here at once that the negative results of Josué were probably due to his having employed insufficient doses of the drug. We used the chemically pure nicotine furnished by Merck in a solution of one to two hundred. It is of importance to bear in mind that the solution, which in order to be effective should be odorless, becomes decomposed after some time and must, therefore, be replaced by fresh solution as soon as the first faint odor of tobacco is noticed. Of this solution one-third cubic centimeter, equivalent to one and one-half milligrams of pure nicotine, was injected into the ear vein of the rabbit every day with the exception of Sundays. It was found dangerous and of no decided advantage to attempt to increase this dosage, which is fully three times that used by Josué and proved entirely satisfactory. About ten seconds after the injection the animal is seized with a violent and quite typical convulsion lasting from about three to five minutes, after which it appears entirely well, eating and feeding quite normally until the next injection, when the same symptoms recur.

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This is repeated with great regularity and without exception with every injection. We have now rabbits that have received over one hundred injections and have never failed to exhibit this daily convulsion with undiminished violence and without the least evidence of any tolerance to the poison being developed. A number of our animals died before they had been injected long enough to produce any definite lesions. In some of these, death ensued from causes not at all referable to nicotine poisoning; in others, however, there were found numerous small infarctions in the lungs for which the intravenous injections were probably responsible. Cerebral hemorrhages, so frequently occurring after intravenous injections of adrenalin, have never been found in our animals. Such animals as outlived a certain number of injections showed distinct and very characteristic lesions. It seems, however, that not all animals are equally susceptible and that individual disposition is an important factor. Thus, in one animal we found well-marked though not very extensive macroscopic lesions in the aorta after only eighteen injections. On the other hand, one rabbit after forty-two injections, another after sixty-seven injections, and a third after one hundred and ten showed no visible abnormalities. These statements, however, refer only to the macroscopic condition of the aorta. The effects of the nicotine injections upon the heart, liver, kidneys, and other organs and tissues are not within the scope of this communication and will be reported later. The lesions produced in the aorta of rabbits by nicotine are very similar to those caused by adrenalin and, according to Fischer, by digalen. In the early stages calcareous patches are found, mainly in the bulb and arch of the aorta. These calcareous areas are irregular in shape and extent; they appear as longitudinal, transverse or slanting ridges, or else in the form of plaques with margins raised above the level of the surface and the center more or less depressed. Sometimes they are quite massive and bulky; at other times and places more delicate and brittle. As the process advances, these chalky areas become more numerous, extending downward over the aorta and involving not only the thoracic but also the abdominal aorta and even the iliacs. Besides these plaques of more or less bulky and rigid calcification, there occur not only in the advanced, but even in the earlier stages, larger or smaller areas in which the wall of the vessel is markedly attenuated and distended, forming genuine distension aneurisms. These aneurismatic dilatations have never been found to involve the entire circumference of the vessel but merely some larger or smaller portion, most frequently the posterior or lateral part of the circumference. In these places the vascular wall is often so thin as to be quite translucent. The inner surface appears entirely smooth and even and no chalky patches are visible, but nevertheless closer examination will detect minute chalky deposits in the depths of the media. The dilatations are found very frequently about the bulb and arch and here in comparatively early stages. Later on they develop along the thoracic and also, though perhaps not so extensively or so often, in the abdominal aorta.

In none of our animals was anything found at all resembling those yellow patches of fatty degeneration so constant and striking in atheroma of the human aorta, though diligent search was made with the aid of Sudan III., and other methods. Neither have we ever seen any gelatinous patches or any evidences of ulceration of the intima. No matter how uneven the inner surface of the vessel becomes through ridges or patches of calcareous deposits, the intima itself, whether raised or depressed, is always found quite smooth and unbroken.

For the histological study of these lesions various methods were employed. After decalcification in five per cent trichloracetic acid the pieces were hardened either in formalin or Zenker's solution, embedded in paraffin and cut in serial sections not thicker than four microns. For staining we used hematoxylin-eosin, safranin, Weigert's elastic stain, Mallory's stain and others.

It can be satisfactorily demonstrated that the earliest change occurs in the muscle cells of the media. As far as we can see only the circular fibers are affected. In all the very numerous sections that we have examined we have never been able to detect the slightest lesion in those muscular layers immediately adjoining the adventitia and which are universally accepted as longitudinal fibers. There is not the least sign of inflammation, nor any evidence of exudate or of leucocyte infiltration. The nucleus of the cell first shows signs of disease, it takes the stain but faintly, becomes irregular in shape, breaks up in scattered bits of chromatin and finally disappears. Then the cell-body itself is affected and gradually transformed into a heap of granular detritus. Take, for example, a transverse section through a part of the aorta immediately adjoining an aneurismatic dilatation (Fig. 1) and which to the naked eye appears entirely normal. Even with a low power it is difficult to find any distinct lesion. The wall of the vessel is of normal thickness, the intima presents its normal appearance as a thin film covering the elastica. All the elastic fibers are regular, showing the normal undulations. A little higher power, however, will show with all distinctness numerous muscle cells in the inner and middle portions of the media in various stages of degeneration, from the first faint affection of the nucleus to the complete destruction of the cell (Fig. 2). As we approach nearer to the distended portion we find the destruction of the cells progressively increasing and their place taken by minute calcareous deposits not visible to the naked eye nor palpable to the finger. When a sufficient number of the muscle cells have been destroyed the elastic fibers seem to lose their wavy, curled appearance and are extended to more or less straight lines. When we finally reach the distended portion of the vessel we find all the muscle fibers gone and their place taken by granular, chalky deposits between the layers of elastic fibers, which are now, evidently under the influence of the blood pressure, which without active support of the muscle cells they are no longer able to withstand, not only completely straightened out, but also distinctly curved with the convexity towards the adventitia and the concavity of the curve towards the lumen of the vessel. The elastica itself does not show a single sign of degeneration and its change of form and arrangement appears purely mechanical. We have seen none of the granular deformity and degeneration described by some authors. The intima, on the other hand, shows distinct signs of proliferation proportionate in magnitude to the amount of depression obtaining in the media (Fig. 3). The structure of the proliferating intima it is difficult to make out. Besides fine elastic material there are great numbers of cells, but whether these are proliferating endothelial cells or connective tissue cells, or possibly smooth muscle cells, and if the latter, whether they correspond to the musculo-elastic layer described by Jores for the human intima, we are at present unable to say.

The process thus briefly sketched represents a type of rather diffuse degeneration and one which leads to attenuation and aneurismatic distension of that portion of the vessel wall affected. In other places a somewhat different type of degeneration occurs, one more circumscribed and localized, and which does not lead to distension but rather to more or less pronounced projection into the lumen in the form of elevated patches and ridges of calcareous material. In this form, also, it is more than probable, though it cannot be so clearly demonstrated as in the preceding type, that the first changes take place in the circular muscle fibers (Fig. 4). These are rapidly destroyed and replaced by more or less bulky, chalky material. The elastic fibers in the immediate neighborhood of these areas of calcification are more or less straightened or deprived of their normal curl (Fig. 5). Those within the region of calcification, however, are in great measure broken and distorted into all manner of irregular pieces and shreds. The breakage of the elastic fibers again impresses one as purely mechanical. There are no signs of degeneration, they take the stain as well as any normal fiber, there is no fine granular detritus, and the edges are always clean-cut and produce a distinct impression of having been forcibly snapped. In the more advanced stages these foci of calcareous degeneration are sometimes, but not always, surrounded by a very slight infiltration of leucocytes which is obviously secondary and due to the irritant effect of the necrotic masses and not at all a part of the original process. In such places where the chalky deposits tend to protrude and bulge forward into the lumen of the vessel (Fig. 6), thus forming those ridges and patches that play so prominent a part in the lesion, it can be seen that the intima covering the protrusion is quite normal. But over the depressed center of the calcareous area, and over the downward slopes of the ridge-like elevations, the intima proliferates vigorously.

Without going into any further details it may be briefly stated that intravenous injections of nicotine produce in the aorta of rabbits lesions of a decidedly necrotic character. What all observers maintain for adrenalin and digalen proves true also for nicotine. It causes neither an endarteritis nor a mesarteritis, but a distinct arterionecrosis affecting primarily and mainly the inner muscular layers of the media. The part played by the elastic tissue seems to be merely a passive one. As long as the muscle cells are fairly normal and active the elastic elements appear perfectly normal. They appear to require, however, the active aid of the muscular fibers to enable them to withstand the strain of the forces acting upon the vessel wall. Thus it comes about that with the failure of the muscular cells and their replacement by chalk deposits the elastic fibers are also incapacitated. They are either stretched to the utmost with apparently complete loss of their elastic qualities, or broken up and mutilated in many ways by the accumulated masses of chalky material deposited among them. The hypertrophy or hyperplasia of the intima appears rather as a reparative process — a strenuous effort to maintain as nearly as may be the normal lumen of the vessel.

Nothing definite can as yet be said as to the nature of the process by which the arterial lesions are affected. It seems most probable to our mind that the drug exerts a toxic influence directly upon the muscle cells, but there are undoubtedly other complicating factors It is not improbable that the interference with the normal blood pressure also plays a part. On the other hand, it is hardly conceivable that the increase of blood pressure alone, transitory as it is and lasting only for a few minutes at a time, even if repeated daily for many months, can be made solely responsible for these lesions. The effect upon the vasa vasorum, the direct irritative action upon the vessel wall, besides other possible factors, must also be considered and much further experimentation will be required to clear up these complex questions.

Can the results thus far obtained be made available for the study of the human vascular lesions and to what extent is it possible to identify these necrotic processes in the aorta of rabbits with the conditions obtaining in arterial sclerosis and aneurism of the human being?

There can be no doubt that we have in intravenous injections of adrenalin, nicotine and digalen a most valuable method for the experimental production and study of aneurism. Many years ago v. Recklinghausen and his pupils (Manchot and others) insisted upon the insufficiency of purely mechanical explanations for aneurism. They maintain that aneurism can occur only in vessels the walls of which are diseased, and they located the primary site of the disease in the media. This is shown now to be absolutely correct. We can trace experimentally the formation of aneurism from the first faint beginnings in the media, visible only under high powers, step by step, to the fully developed aneurismatic pouch with highly attenuated wall and almost complete obliteration of the muscular and elastic elements. We can see, too, that the most important element in the media upon which its function and with it that of the artery mainly depends is not the elastica, which plays merely a secondary part, but the muscular element.

In comparison with human arteriosclerosis, however, the facts are not quite so obvious. The gross appearance of the lesions in the aorta of rabbits, especially in the more advanced stages, undoubtedly shows a striking similarity with human atheroma. There are evident, however, upon closer examination, many points of difference. It has been dwelt upon by nearly all investigators with adrenalin that while in human arteriosclerosis, at any rate in the more advanced stages, the process is widely disseminated over the whole arterial system, the experimental lesions are strictly limited to the aorta. Erb is the only one who seems to have found lesions in other arteries. Nicotine appears to constitute no exception to this rule. Thus far we have not been able to detect arterial disease anywhere but in the aorta. This, however, is not a very grave objection. As the experiments are continued we may hope to find lesions in other arteries also, and it must not be forgotten, as Pearce and Stanton[8] have pointed out, that our experimental lesions are, after all, an acute process, the results of at most a few months, while human arteriosclerosis is a process developing in the course of years. Besides, as is well known, the arteriosclerotic process in man is by no means always so generally disseminated. Often enough it is only the aorta that is affected. Often again it is merely one or the other peripheral arterial territory. For the present, therefore, only the aortic lesions can be compared, and here we find some notable differences. The fatty degeneration — the yellow patches invariably found in every case of human atheroma which most observers locate in the superficial layers of the intima, but which, as Jores has shown, extend much further down into the wall of the vessel, are never found in the aorta of rabbits. The same holds good for the so-called gelatinous patches. Great stress has also been laid upon the extensive hyperplasia of the intima in the human being, which is said to be entirely wanting in the experimental lesions in the rabbit. For these reasons nearly all investigators abroad and many in our own country are inclined to the opinion that these experimental lesions in the rabbit are a specific arterionecrosis and have nothing in common with human arteriosclerosis.

To us this extreme view does not appear warranted by the facts. It would be most premature at this early stage to speak with any kind of assurance upon so complicated a question. The entire theory of human arteriosclerosis is at the present moment in one of those periods of transition in which a multitude of different views are competing and it would tax the ingenuity of the most learned pathologist just now to furnish a definition of arteriosclerosis that would be completely satisfactory. The older theories according to which arteriosclerosis proper is some form of inflammatory process, whether of the intima, as a proliferating endarteritis or of the media, as a mesarteritis or of both together, are decidedly on the wane. The brilliant researches of Thoma and his pupils, though they have contributed much to the advancement of our knowledge, have not been able to furnish a complete explanation of all the facts of arteriosclerosis. Thoma himself was compelled to postulate some lesion of the media to make his theory fit. The best and most authoritative of recent opinions tends more and more towards the conviction that human arteriosclerosis is primarily a degenerative process; thus Jores in his painstaking and thorough work, so also Marchand[4] in his elaborate report on arteriosclerosis.

The main difficulty seems to be in fixing the point of origin of the degenerative process and tracing it from its inception to the final stages. It is obvious that for this purpose the histological study of the human arteries alone must be entirely insufficient, and the great amount of experimental work that has been essayed until now has advanced us but little. Much of it, in fact, has tended rather to obscure and confuse the questions involved than to clear them up. It is here that the new method of intravenous injections of adrenalin, nicotine, and no doubt a great many other substances still to be tested, promises much enlightenment.

It may not after all be very difficult to show that the nicotine lesions in the rabbit's aorta, and of course this holds good also for adrenalin, do not differ so very widely from the aortic lesions in human atheroma. It is necessary to bear in mind the difference in structure that exists between the human and the rabbit's aorta. In the normal aorta of the rabbit, the intima is but a thin film consisting of a layer of endothelium lying almost immediately upon the elastica interna with but a few insignificant cells of undefined character between. The human intima is a much more complicated structure which is supposed to contain muscular and elastic elements to such an extent that it appears at times not clear where the intima terminates and the media begins. The elastica itself is much more delicate and contains none of the massive elastic plates which are a regular constituent of the human aorta. These facts alone will help us to understand the absence of the patches of fatty degeneration in the rabbit. We must consider, furthermore, the differences in metabolism that exist between an herbivorous rodent and an omnivorous man. Hansemann[5] has pointed out that rabbits, as all herbivorous animals, tend much more to chalky deposits and calcifications than man. On the other hand, Moenckeberg[6] collected numerous cases of calcification almost entirely confined to the media in human arteries, not so much in the aorta as in the larger arteries of the extremities, and leaves no doubt that they are connected with the arteriosclerotic process. While thus some of the more notable differences in the two conditions may be explained, the striking similarities are obvious. We have the extensive elevated patches and plaques of calcification, the aneurismatic distension and the secondary hypertrophy of the intima, which latter, considering the delicacy of the normal intima of the rabbit, assumes under these abnormal conditions really huge proportions, fully comparable, we are inclined to think, to the arteriosclerotic proliferations in man.

From all this it may perhaps be concluded that the adrenalin and nicotine lesions in the aorta of rabbits and the lesions of human arteriosclerosis, if not strictly identical, are at least to a great extent analogous. Further experimental study by means of the intravenous method will, it may be confidently expected, help us to a better understanding of the complicated pathology of human arteriosclerosis.

We desire to express our thanks to Prof. J. G. Curtis, through whose kindness our numerous rabbits were accorded the hospitable care of the physiological laboratory of the College of Physicians and Surgeons during the last six months. We are indebted to Dr. Herman M. Adler for the photographic illustrations.

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DESCRIPTION OF PLATES.

Plate XXI. — Fig. 1. Transverse section of aorta after forty-seven injections of nicotine. Very low power. To the left apparently normal, to the right highly attenuated and distended portion of the wall. The black strip indicates calcareous deposit in the media. Hematoxylin-eosin stain.

Fig. 2. Same section. Portion of the apparently normal part under high power (Zeiss apochromatic, eight millimeters) showing disseminated areas of cell necrosis.

Fig. 3. Same section; same power. Distended portion of the vessel showing calcification in the media, stretching of the elastic fibers and proliferation of the intima.

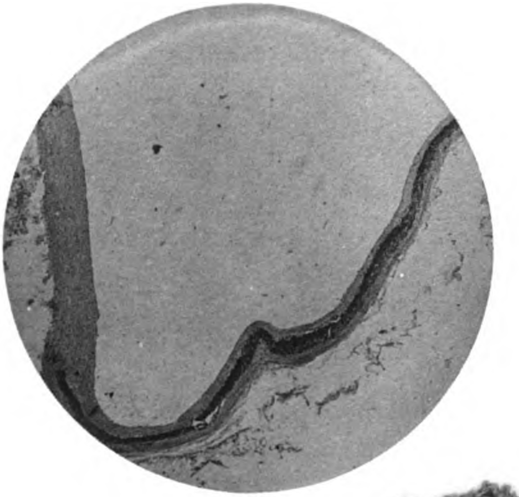
Plate XXII. — Fig. 4. Aorta after fifty injections. (Zenker's solution, safranin stain; Zeiss apochromatic, eight millimeters.) Shows cell necrosis and incipient disturbance of the elastica by calcification. The larger empty light spaces show where chalk had been deposited before decalcification.

Fig. 5. Cut from the same aorta. Shows large patch of calcification surrounded by some leucocyte infiltration, stretching of the elastic fibers and considerable hypertrophy of the intima.

Fig. 6. Same aorta; same stain; same power. A very massive area of calcification forming a ridge in the lumen. Shows the mutilation and deformation of the elastic fibers and the proliferation of the intima at either side of the elevation.

Plate XXIII. — Figs. 7, 8, 9. Same aorta. Variouss tages of calcification, stretching and breaking of the elastica. In Fig. 7 proliferation of the intima.

Fig. 10. Same aorta. (Zenker's solution, safranin stain; Zeiss apochromatic, three millimeters.) Various stages of necrosis of the muscle cells.



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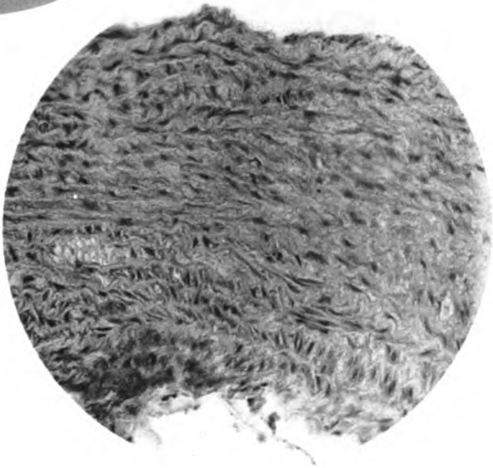

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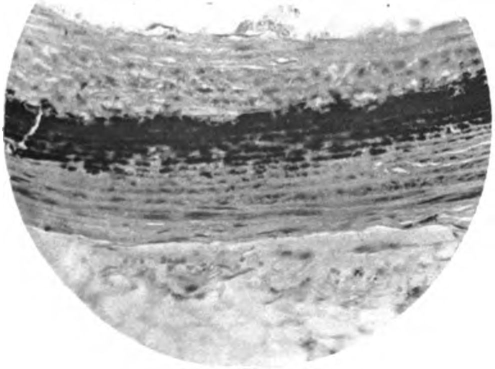

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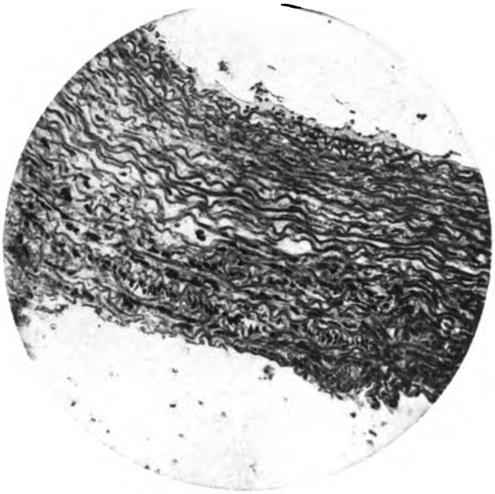
Adler and Hensel.

Nicotine injections.



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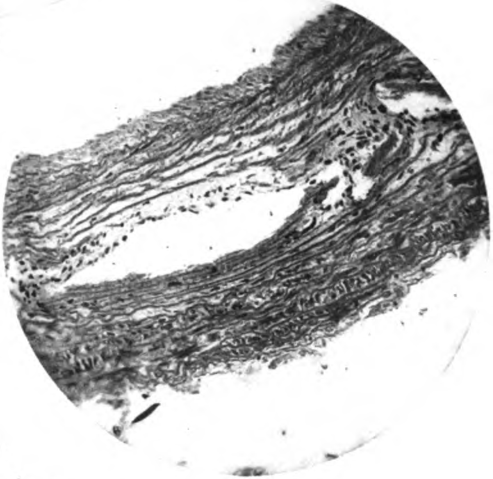

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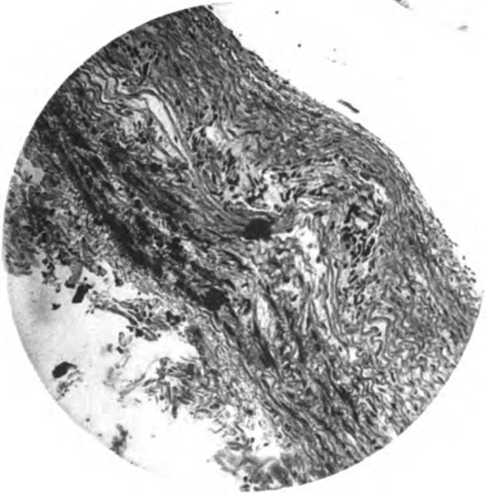

6

Adler and Hensel.

Nicotine injections.

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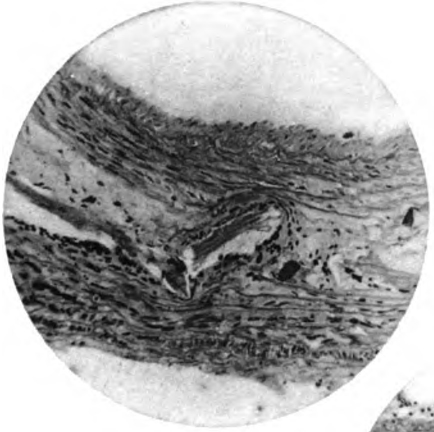

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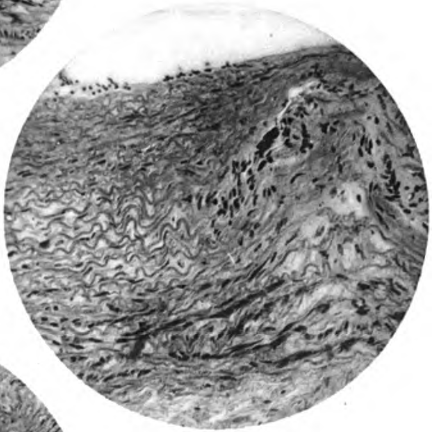

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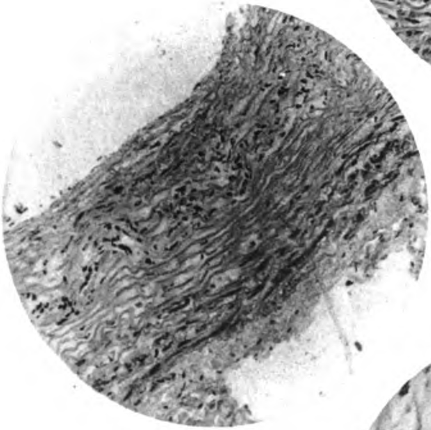

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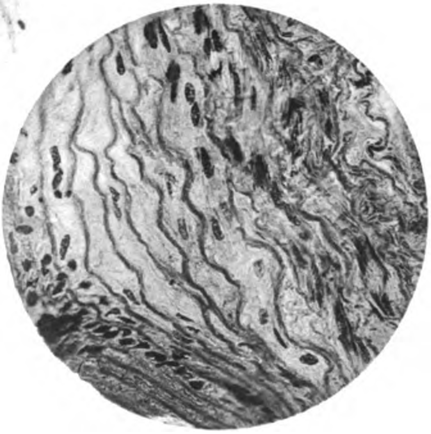

10

Adler and Hensel. Nicotine Injections.

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TRANSMISSION OF RESISTANCE TO DIPHTHERIA TOXINE BY THE FEMALE GUINEA-PIG TO HER YOUNG.*

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When the Hygienic Laboratory undertook the work of preparing a standard unit for the measurement of antidiphtheritic serum one of the first necessities was a constant and reliable supply of guinea-pigs weighing from two hundred and fifty to two hundred and eighty grams. It was estimated that five hundred females in the breeding pens would insure a sufficient number for this as well as for the other work of the laboratory. It occurred to me that perhaps we might use as breeders the pigs that had been used for testing antitoxine or determining the strength of diphtheria toxine and had fully recovered. Before placing such pigs in the breeding pens I thought it would be well to first test the young of a number of such used and recovered pigs to see if they were resistant to the action of diphtheria toxine. For this purpose twenty-one pigs were placed aside and their young tested either by the giving of a MLD or L+ dose, controls being used in every case from the stock pigs whose mothers previously had had no treatment. All of the animals were under exactly the same conditions as regards food, temperature, air, and light, both before and after testing.

The determination of this question is of threefold importance.

First, we know from work by Rosenau and Anderson[1] that there is a hypersusceptibility to the injection of horse serum, normal or antitoxic, transmitted by a mother pig which has had a toxine-antitoxine mixture or serum alone to her young. It is, therefore, manifest that young pigs from such mothers should not be injected with serum for the purpose of determining the absence of bacterial contamination. In this test

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* Received for publication July 5, 1906.

it is usual to inject several cubic centimeters of the serum under examination, and in the work referred to one cubic centimeter was sufficient to cause the death of pigs with a hypersusceptibility transmitted from the mother.

Second, if treated-and-recovered pigs are used as breeders and their young prove more resistant to diphtheria toxine, then when tests of serum are made on such resistant pigs we would evidently obtain misleading results.

Third, if these young pigs are of average susceptibility the recovered pigs could be used as breeders, resulting in quite a saving in animals bought for breeding purposes.

The use of such used animals as breeders was the custom in two antitoxine laboratories that I personally know of and I suggested it as one explanation of irregularities they encountered in the testing of antitoxine. Both eliminated these used animals from their breeding pens with a decided decrease in the number of irregular results.

No pigs have been bought for use in the Hygienic Laboratory since May thirty-first, 1905, the number raised being ample. We obtained pigs from three sources for breeding purposes, by far the larger number being presented to the laboratory by the Bureau of Animal Industry, none of which, I am sure, for many generations back had any taint of diphtheria toxine. As to the other two sources of supply I cannot be sure; but all of those received at the laboratory were under three hundred grams in weight and consequently would not have been used for work with diphtheria toxine. Since May, 1905, we have used three thousand four hundred and thirty-nine pigs for the testing of diphtheria toxine or antitoxine. During this time a careful search of the records shows a very small number of pigs which died much earlier than was to be expected with the dose given and some of these presented pneumonia at the autopsy. Only three have failed to die acutely when used on the L+ dose, thus showing very plainly that marked resistance to diphtheria toxine is unusual in the pigs bred at the Hygienic Laboratory.

Theobald Smith,[2] in giving the results of tests of the young of three female pigs in which there was an immunity transmitted from the mother, says that this may be due to (1) the influence of any primary treatment, (2) the influence of the male, (3) the influence of selection in breeding. He considers his data sufficient to rule out the first, but states that the mother of his most resistant pigs (No. 2944) had previously been treated with a toxine-antitoxine mixture and about four or five months afterwards put into the breeding pens. I think, in view of my results, that this treatment accounts for the immunity of her young.

In regard to his third statement I think that the results obtained at the Hygienic Laboratory with the pigs raised here show that in our guinea-pigs, at least, insusceptibility to diphtheria toxine is very rare. I am inclined to think that the influence of artificial selection in breeding is of slight importance if previous treatment of the breeders with the diphtheria toxine-antitoxine mixture can be excluded for several generations back. I can conceive that by the selection of breeders who have shown a special resistance to diphtheria toxine a very resistant race of pigs may be raised.

It is not improbable that dealers and breeders of guinea-pigs may buy at times used guinea-pigs from persons engaged in work with diphtheria antitoxine and thus introduce unknowingly toxine-resisting pigs into their stock.

The[1] first experiments to immunize animals during pregnancy against pathogenic bacteria were positive. Chauveau found that the young of sheep so immunized were immune to anthrax, and like results were arrived at by Arloing, Cornevin and Thomas in symptomatic anthrax.

Ehrlich was the first to study the problem methodically, and his results have been confirmed and stand. He used mice that had acquired a high degree of antitoxine immunity through systematic feeding of ricin, abrin, and robin. He showed that the male (sperm) element was incapable of transmitting the immunity.

Ehrlich used female animals that were immune before becoming pregnant. By using animals that were being immunized during pregnancy his results were interpreted as negative, inasmuch as positive results could not be referred to an active intrauterine immunization of the fetus. In all of these cases his results were positive in that about four weeks after birth a definite immunity was demonstrable. About one and a half months after birth there still remained some undoubted immunity, but in the course of three months all traces had disappeared.

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[1] NOTE. — The following summary of a part of the literature upon the influence of heredity in immunity is largely taken from the article by Morgenroth in Kolle and Wasserman's Handbuch der pathogenen Mikroörganismen, iv, 784. Those interested will find this an excellent discussion of the subject with bibliography.

This short immunity in the progeny of mothers was considered as passive immunity, and rested on the transmission of antibodies from the mother. Against inheritance in the strict sense there was the fact that there was absolutely none present in the grandchildren of immune mothers.

Ehrlich, therefore, concludes that neither sperm nor germ transmits the immunity, and so no inheritance in the strict sense takes place.

Ehrlich, by his "mother-exchange" experiment, showed that the antitoxines furnished in intrauterine life did not long (twenty-one days) remain in the young organism. He showed positively that the milk was the vehicle by which the suckling organism received its antitoxine, and with the length of the suckling furnishes it with an increasing immunity. The long persistence of the immunity depended upon a transfer of antibodies through the milk. By the immunization of a nursing mother mouse (after birth of the litter), Ehrlich was able to transmit immunity to swine-plague to the nursing young.

Ehrlich and Hübener showed that Tizzoni and Centani had erred when they appeared to show that in rabbits the progeny of a male immune to hydrophobia, and a female immune to tetanus was immune to hydrophobia. In experiments with tetanus-immune guinea-pigs and mice, they obtained results in entire harmony with the previous results of Ehrlich; and, opposed to Tizzoni's reports, established that in tetanus also no transmission occurs through a male, but that only through the mother does transmission of immunity take place which, by the end of the second and positively by the end of the third month, disappears.

Vaillard came to the same conclusions from experiments on guinea-pigs and rabbits.

The results of Wernicke on diphtheria-immune guinea-pigs were also the same. "In diphtheria the immunity is not transmitted by the father, but the mother only." This immunity cannot be detected in the grandchild, but remains longer in the first generation (the children), for in three months a considerable immunity is still present.

Transmission of immunity through the milk also occurs in guinea-pigs.

Klemperer found in eggs of tetanus-immune hens tetanus antitoxine in the yolk, not in the white.

Kitt injected hens with eggs derived from chicken cholera-immune hens and obtained immunity.

Sclavo immunized hens against diphtheria by injection of weakened cultures, and found that the whites of the eggs protected guinea-pigs against lethal doses of diphtheria bacilli.

Tables Nos. I. and II. show the results of tests of the young of female guinea-pigs that had recovered from the effects of an injection of toxine alone or of the toxine-antitoxine mixture. These animals were put aside for breeding after having been under observation thirty-five days after injection, which is the time all animals used for antitoxine work in the laboratory are kept under observation.

Table No. I. shows the results of the tests of the young pigs against an L + dose, and Table No. II. against an MLD.

TABLE NO. I.

Showing resistance to the L+ dose in guinea-pigs born of a mother treated with a single injection oj toxine or toxine-antitoxine mixture.

MOTHERS.							YOUNG PIGS.						CONTROLS.	
No.	Treatment.				Result.		No.	Dose.		Result.			Result.	
	Toxine.		Serum.		Reaction.	Paralysis.		Toxine.	Serum.	Death.	Recovery.			
	No.	Amt.	No.	Amt.							Reaction.	Paralysis.	Death.	Recovery.
608....	29a	.002	0	0	Ulcer.	—	608A ..	.22	1 i.u.	3-22			2-19	
1424....	9	.12	Ehr.	1 i.u.	—	—	1424A ..	.22	1 i.u.	8-3			5-7	
							1424B ..	.22	1 i.u.	7-2			5-0	
1992....	9	.23	A4	1 i.u.+	—	—	1992A ..	.22	1 i.u.	....	—	—	2-6	
1524...	7	.21	B14	1 i.u.+	—	+	1524C...	.22	1 i.u.	....	Ulcer.	—	2-1	
1909....	5	.133	140	$\frac{1}{400}$	—	—	1909A ..	.22	1 i.u.	2-15			2-15	
1773....	17A	.006	0	0	Ulcer.	—	1773A ..	.22	1 i.u.	2-17			3-7	
1909 ...	5	.133	140	$\frac{1}{400}$	—	—	1909B ..	.22	1 i.u.	3-16			2-22	
608....	29a	.002	0	0	Ulcer.	—	608E ..	.22	1 i.u.	3-8			2-12	
1907....	5	.133	137	$\frac{1}{200}$	—	—	1907A ..	.22	1 i.u.	3-3			3-3	
1946....	15A	.3	B17	1 i.u.	—	—	1946C...	.22	1 i.u.	....	Induration.	—	3-3	
							1946A ..	.22	1 i.u.	....	Induration.		2-17	
							1946B ..	.22	1 i.u.	....	Ulcer.	—	3-0	

1766....	17A	.005	0	0	Ulcer.	—	1766A ..	.22	1 i.u.	3-0			3-0
							1766B ..	.22	1 i.u.	2-19			3-0
1902....	5	.133	184	$\frac{1}{600}$	—	—	1902A ..	.22	1 i.u.	....	Ulcer.	+	2-12
							1902B ..	.22	1 i.u.	....	Ulcer.	+	2-12
1905....	5	.133	137	$\frac{1}{200}$	—	—	1905A ..	.22	1 i.u.	....	Ulcer.	+	3-0
2264 ...	19	.007	0	0	Ulcer.	+	2264C...	.22	1 i.u.	2-14			2-12
3827....	7	.22	201	$\frac{1}{300}$	—		3827C...	.22	1 i.u.	....	Ulcer.	—	3-2
608....	29a	.002	0	0	Ulcer.	—	608B ..	No. 15, .48	1 i.u.	2-23			2-3
1524....	7	.21	B14	1 i.u.+	—	—	1524A ..	No. 15, .49	1 i.u.	....	Ulcer.	+	2-21
							1524B ..	No. 15, .49	1 i.u.	7-5			2-21
1277....	5	.130	IX-17	1 cc.	—	—	1277B ..	No. 15, .50	1 i.u.	3-20			3-6

A study of this table shows that of fourteen mothers, seven, or fifty per cent, transmitted an immunity to their young sufficient to withstand the effects of an L + dose; all living, except Nos. 1424A, 1424B, and 1524B, which lived three, two, and four days respectively longer than their controls. Of a total number of twenty-four young pigs shown in this table, thirteen, or 54.16 per cent, were resistant to an L + dose.

Of the mothers that received the toxine-antitoxine mixture, seven, or seventy per cent, transmitted an immunity to their young.

The fact that no mother which received a dose of toxine alone transmitted an immunity to her young is very plainly shown.

TABLE NO. II.

Showing resistance to the MLD of guinea-pigs born of a mother treated with a single injection of toxine or a toxine-antitoxine mixture.

MOTHERS.							YOUNG PIGS.						CONTROLS.	
No.	Treatment.				Result.		No.	Dose.		Result.			Result.	
	Toxine.		Serum.		Reaction.	Paralysis.		Toxine.	Serum.	Death.	Recovery.			
	No.	Amt.	No.	Amt.							Reaction.	Paralysis.	Death.	Recovery.
608....	292	.002	0	0	Ulcer.	—	608F ...	No. 9, .005	0	2-5			2-18	
1278....	5	.130	IX-17	$\frac{1}{100}$	—	—	1278C...	No. 9, .005	0	....	Ulcer.	+	4-1	
1907....	5	.133	137	$\frac{1}{100}$	—	—	1907B ..	No. 9, .005	0	....	Ulcer.	—		
1277....	5	.130	IX-17	1 cc.	—	—	1277A...	No. 9, .006	0	3-12			2-13	
1440....	31B	.006	—	—	Indurated.	—	1440B...	No. 9, .006	0	2-10			2-5	
1445....	34A	.006	0	0	Ulcer.	—	1445A ..	No. 9, .006	0	2-8			2-21	
608....	29A	.002	0	0	Ulcer.	—	608D ...	No. 15, .01	0	4-0			3-7	
1278....	5	.130	IX-17	$\frac{1}{100}$	—	—	1278A ..	No. 15, .01	0	....	Ulcer.	+	3-19	
1440....	31B	.006	—	—	Indurated.	—	1440A ..	No. 15, .01	0	2-21			5-3	
1773....	17A	.006	—	—	Ulcer.	—	1773B...	No. 15, .01	0	4-23			4-9	
1445....	34A	.006	0	0	Ulcer.	—	1445B ..	No. 15, .02	0	2-7			2-5	
1277....	5	.130	IX-17	1 cc.	—	—	1277C...	No. 15, .02	0	2-12			2-12	
1278....	5	.130	IX-17	$\frac{1}{100}$	—	—	1278B ..	No. 15, .02	0	....	Ulcer.	+	2-12	

TABLE NO. II. — *Continued.*

MOTHERS.							YOUNG PIGS.						CONTROLS.	
No.	Treatment.				Result.		No.	Dose.		Result.			Result.	
	Toxine.		Serum.		Reaction.	Paralysis.		Toxine.	Serum.	Death.	Recovery.			
	No.	Amt.	No.	Amt.							Reaction.	Paralysis.	Death.	Recovery
1426....	9	.16	Ehr.	1 i.u.	—	—	1426A ..	No. 7, .006	0	4-0			3-3	
1427....	9	.18	Ehr.	1 i.u.	—	—	1427B ..	No. 7, .006	0	....	Ulcer.	—	2-17	
1497....	5	.130	B14	1 i.u.+	—	+	1497A ..	No. 7, .006	0	....	Ulcer.	—	2-22	
							1497B ..	No. 7, .006	0	....	Ulcer.	—	3-8	
1905....	5	.133	137	$\frac{1}{200}$	—	—	1905B ..	No. 7, .006	0	....	Ulcer.	+	3-6	
							1905C...	No. 7, .006	0	....	Ulcer.	+	3-6	
4495....	7	.19	B27	1 i.u.	+	+	4495A ..	No. 7, .007	0	....	—		2-2	
							4495C...	No. 7, .007	0	....	—		1-12	

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An examination of this table shows that of twelve mothers, six, or fifty per cent, transmitted an immunity to their young.

Of a total of twenty-one young pigs treated, eleven, or fifty-two per cent, were resistant to a fatal dose for control pigs.

Of the mothers that received the toxine-antitoxine mixture seventy-five per cent transmitted an immunity to their young.

No mother, that had received an injection of toxine alone, transmitted this immunity.

It will be noticed in both tables that where an immunity is transmitted to the young all of the litter are immune to about the same degree.

The question will naturally be asked, Why is this immunity not transmitted by all the mothers treated with the toxine-antitoxine mixture? I cannot answer this question positively, but suggest that it may be due to the fact that some of the pigs produce more antitoxine than others. It is a well-known fact among producers of diphtheria antitoxine that some horses can never be made to produce a serum of even two hundred and fifty units, which is considered a rather low potency serum. Such may be the case with guinea-pigs.

The following data, kindly furnished me by Dr. J. J. Kinyoun, is of interest in this connection. It will be seen by reference to the table (No. III.) that some horses, in spite of prolonged treatment, never produce any antitoxine.

TABLE NO. III.

Table showing that some horses, in spite of prolonged treatment, fail to produce any antitoxine or only small amounts.

No.	Duration of Treatment.	No. MLD for 250 gm. G. P.	No. of Trials.	Highest Value.	Result.
1.	14 weeks.	1,280,000	4	0 units.	Died.
2.	18 "	1,110,000	5	50 "	"
3.	19 "	1,010,000	4	100 "	"
4.	20 "	2,080,000	5	100 "	"
5.	20 "	1,800,000	3	100 "	"
6.	22 "	1,520,000	5	0 "	Returned.
7.	22 "	2,390,000	10	100 "	"
8.	24 "	1,980,000	6	0 "	Died.
9.	25 "	2,810,000	12	100 "	"
10.	25 "	2,590,000	5	0 "	Transferred.
11.	25 "	3,990,000	8	0 "	"
12.	26 "	5,710,000	12	100 "	Died.
13.	28 "	3,520,000	10	100 "	Returned.
14.	30 "	3,770,000	6	100 "	Died.
15.	32 "	4,600,000	12	0 "	Transferred.
16.	36 "	5,530,000	11	50 "	Died.
17.	36 "	4,000,000	15	100 "	"
18.	37 "	4,500,000	14	100 "	Returned.
19.	39 "	6,030,000	9	100 "	Died.
20.	47 "	19,500,000	23	150 "	Transferred.
21.	48 "	10,560,000	20	150 "	"
22.	48 "	9,090,000	17	100 "	Returned.

Attention is called especially to horses Nos. 1, 6, 8, 10, 11, and 15, which never produced serum with antitoxic properties.

THE EFFECT OF REPEATED INJECTIONS IN THE MOTHER ON HER YOUNG. — At the suggestion of Dr. Rosenau a number of female guinea-pigs were given repeated injections either of antitoxine alone or of one unit of antitoxine plus slightly more than the L° dose of toxine in order to study the effects of repeated injections in the mother on her young. An untreated male was placed in the pen with them. The young were allowed to remain with their mother until the day before being tested. They were tested in most cases against an L+ dose, though in some cases a larger dose, either one and a third or one and a fourth times the L+ dose, was given.

Pigs Nos. 4E, 4F, 8G, and 8H received 8⅛ times the MLD.

GUINEA-PIG NO. 4.

May 25, 1905. Given .15 cubic centimeter toxine No. 7 (L+ .21) + 1 immunity unit Ehrlich 15.IV.05.

May 29. Same treatment.

June 9. .18 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

June 16. .19 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

June 26. .19 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

July 12. .19 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15 IV.05.

Litter No. 1.— July 28, 1905. 4A and 4B born.

August 25. 4A given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VIII.05. Moderate reaction at site on third day. Recovered. Control died in 3 days, 3 hours.

August 25. 4B given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VIII.05. Died in 6 days, 3 hours. Control died in 3 days, 3 hours.

Litter No. 2. — Nov. 7, 1905. 4C and 4D born.

Jan. 6, 1906. 4C given .22 cubic centimeter toxine No. 7 + 1 immunity unit B27. No reaction. Control died in 3 days, 7 hours.

January 24. 4D given .293 cubic centimeter toxine No. 7 (= 1⅓ times the L+ dose). Induration followed by small ulcer. Recovered. Control died in 1 day, 15 hours.

Litter No. 3. — May 21, 1906. 4E and 4F born.

June 22. Both given .05 cubic centimeter toxine No. 7 (MLD = .006 cubic centimeter). Slight induration third day. Controls died in 1 day, 4 hours, and 22 hours respectively.

GUINEA-PIG NO. 5.

Same treatment as No. 4.

Litter No. 1. — July 27, 1905. 5A and 5B born.

September 9. Both given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IX.05. Slight induration at site in both pigs. Recovered. Controls died in 3 days, 12 hours.

Litter No. 2. — Oct. 27, 1905. 5C and 5D born.

Jan. 6, 1906. Both given .22 cubic centimeter toxine No. 7 + 1 immunity unit B27. No reaction in either pig. Controls died in 3 days, 7 hours.

Litter No. 3. — Feb. 3, 1906. 5E born.

March 17. Given .293 cubic centimeter toxine No. 7 (1⅓ times the L+ dose) + 1 immunity unit B28. Very slight reaction fourth day. Recovered. Control died in 1 day, 18 hours.

Litter No. 4. — April 12, 1906. 5F and 5G born.

June 5. Both given .275 cubic centimeter toxine No. 7 (1¼ times the L+ dose) + 1 immunity unit Ehrlich 15.VI.06. Both showed induration followed by ulcer at site. Control died in 1 day, 21 hours.

GUINEA-PIG NO. 6.

Same treatment as No. 4.

Litter No. 1. — Sept. 7, 1905. 6A and 6B born.

October 20. Both given .22 cubic centimeter toxine No. 7 + 1 immunity unit B25. No reaction. Controls died in 3 days, 2 hours.

Litter No. 2. — Nov. 8, 1905. 6C, 6D, 6E, and 6F born. 6C, 6E, and 6F used for other work.

Jan. 24, 1906. 6D given .293 cubic centimeters toxine No. 7 (1⅓ times the L+ dose) + 1 immunity unit B27. Small ulcer. Recovered. Control died in 1 day, 20 hours.

Litter No. 3. — Feb. 17, 1906. 6G born.

March 17. Given .293 cubic centimeter toxine No. 7 (1⅓ times the L+ dose). Very slight reaction. Control died in 1 day, 18 hours.

Litter No. 4. — April 26, 1906. 6H and 6I born.

June 5. Both given .276 cubic centimeter toxine No. 7 (1¼ times the L+ dose). No reaction in either pig. Control died in 1 day, 21 hours.

GUINEA-PIG NO. 7.

May 25, 1905.	Given	250	units	antitoxine.
June 9.	"	600	"	"
June 16.	"	900	"	"
June 26.	"	1,000	"	"
July 12.	"	1,500	"	"

Litter No. 1. — July 26, 1905. 7A born.

August 16. Given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VIII.05. Small ulcer. Recovered. Control died in 3 days, 7 hours.

August 24. The mother was killed, blood drawn, allowed to clot, and serum tested to 12, 25, and 50 units. All the pigs died in about 24 hours; showing that her serum contained considerably less than 12 units per cubic centimeter.

GUINEA-PIG No. 8.

Same treatment as No. 7.

Litter No. 1.— July 29, 1905. 8A, 8B, and 8C born. 8B and 8C died.

September 15. 8A given .22 cubic centimeter toxine No. 7 + 1 imunity unit Ehrlich 15.VIII.05. Died in 7 days, 7 hours. Control died in 4 days.

Litter No. 2.— March 15, 1906. 8D, 8E, and 8F born.

April 27. 8D given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VIII.06. No reaction. Control died in 4 days, 8 hours.

April 27. 8E and 8F given same injection as 8D. Both died on the third day. Controls died on the third day.

Litter No. 3. — May 20, 1906. 8G, 8H, and 8I born.

June 22. 8G and 8H given .05 toxine No. 7 (MLD = .006 cubic centimeter). 8G died in 1 day, 4 hours. Control died in 22 hours. 8H showed slight induration on third day. Recovered. Control died in 1 day, 4 hours.

A study of the above results shows that the young of all the mothers that were treated by repeated injections of the toxine-antitoxine mixture had a marked resistance to an L+ dose; all survived except one, 4B, which died, but lived three days longer than the control. Four (4D, 5E, 6D, and 6G) withstood one and a third times the L+ dose; this dose uniformly caused the death of the controls in less than two days. Four (5F, 5G, 6H, and 6I) resisted one and a fourth times the L+ dose.

Of four pigs tested against eight and a third times the MLD, three recovered (4E, 4F, and 8H); one (8G) showed the average susceptibility to the toxine.

The members of the fourth litter are as resistant as those of the first. The immunity seems to be greater in those pigs born of mothers treated with the toxine-antitoxine mixture than in those treated with the antitoxine alone. Pigs Nos. 4, 5, and 6 received a total of only six units, given in doses of one unit plus an amount of toxine slightly greater than the L^0 dose, while Pigs 7 and 8 received 4,250 units. Of seven young of these last two mothers, three recovered, one lived three days longer than the control, and three showed no resistance.

Moreover, it would seem that the immunity produced by the injection of large amounts of antitoxine alone is not as lasting as that produced by small amounts plus small doses of toxine.

PERSISTENCE OF THE IMMUNITY.—Two pigs, born November eighth, 1905, of Pig No. 6, were placed aside to determine how long the immunity transmitted from the mother lasted. One member of the same litter (6D) resisted one and a third times the L+ dose, and all of the young of this mother from four litters have shown a marked resistance to the toxine. The results are as follows:

Guinea-pig No. 6C. Weight 650 grams, age 226 days. Given .293 cubic centimeter toxine No. 7 + 1 immunity unit B29. Died in two days, twenty hours.

Guinea-pig No. 6D. Weight 510 grams, age 226 days. Given .293 cubic centimeter toxine No. 7 + 1 immunity unit B29. Died in two days, two hours.

Controls for the above pigs died in two days, three hours, and two days, four hours respectively.

It will be seen that there was no difference in the resistance of the test pigs and the controls. It is, therefore, evident that the immunity had entirely disappeared in seven months.

IMMUNITY NOT TRANSMITTED TO SECOND GENERATION.—Two female guinea-pigs born of mother No. 6, and from a litter of which the other members had shown marked resistance to diphtheria toxine, were placed with a male pig born of untreated parents. Each female had two young. These were given an L+ dose to determine whether the immunity was transmitted to the second generation (grand-children). The results are given below:

Guinea-pig No. 6ac. Given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VI.06. Died in three days, fourteen hours. Control died in three days, seventeen hours.

Guinea-pig No. 6bc. Given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VI.06. Died in three days, twenty hours. Control died in three days, fourteen hours.

Guinea-pig No. 6aa. Given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VI.06. Died in four days, four hours. Control died in three days, seventeen hours.

Guinea-pig No. 6ba. Given .22 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.VI.06. Died in five days, seventeen hours. Control died in three days, fourteen hours.

From the above it seems plain that the resistance to diphtheria toxine is not transmitted to the second generation.

THE CUMULATIVE EFFECT OF DIPHTHERIA TOXINE. — An attempt was made to immunize female guinea-pigs by repeated small injections of diphtheria toxine. Doses considerably less than the MLD and not sufficient to cause a perceptible reaction were given at intervals of about seven days. Before an amount equal to an MLD had been given the animal in each case died. These results are in exact accord with the work of von Behring and Kitashima,[8] who found that guinea-pigs, when given daily injections of diphtheria toxine, after a time, and when the total amount injected was only $\frac{1}{400}$ of an MLD, died, with the post-mortem lesions of poisoning from diphtheria toxine. Instead of being able to produce an immunity to the toxine a hypersusceptibility was produced.

A study of Tables I. and II. will show that in no case in which the preliminary treatment of the mother was of toxine alone was there an immunity transmitted by the mother to her young. This at first seemed to me a remarkable fact. I conceive the reason of the failure of this immunity to be transmitted by pigs whose preliminary treatment was toxine alone to be due to the fact that the mother pig was not immunized by a single dose of toxine, and that it is difficult to produce an active immunity in guinea-pigs by the repeated injections of toxine; for, before this result is obtained, the cumulative effect of the toxine kills the animal. It seems to me that instead of calling it a hypersusceptibility to the poison, as did von Behring and Kitashima, it would be more proper to consider this action due to the cumulative effect of repeated small doses of the toxine.

It is well known among clinicians that one attack of diphtheria, rheumatism or pneumonia predisposes to a subsequent one. This may more properly be considered due to an induced hypersusceptibility, whereas the repeated injections of toxine, as in the case of the guinea-pigs, is a cumulative action.

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SIMULTANEOUS TRANSMISSION OF RESISTANCE TO DIPHTHERIA TOXINE AND HYPERSUSCEPTIBILITY TO HORSE SERUM BY THE FEMALE GUINEA-PIG TO HER YOUNG.*

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We know that the young of female guinea-pigs that have received, either before or during pregnancy, toxine-antitoxin mixtures are hypersusceptible to an injection of horse serum either normal or antitoxic. It therefore occurred to me that it would be very interesting to determine whether such young pigs were also *immune* to diphtheria toxine.

For this purpose it was necessary first to test the immunity against the MLD, and if the animal recovered to test its hypersusceptibility to horse serum, as treatment with toxine alone does not render a guinea-pig susceptible to a subsequent injection of horse serum.

Guinea-pigs born of a susceptible mother and from a litter of which the other members were immune to diphtheria toxine were also used to test the transmission of hypersusceptibility to horse serum. The results are as follows:

FAMILY No. 1.—Mother guinea-pig No. 4495. 103 days before the birth of young given .19 cubic centimeters toxine No. 7 + 1 immunity unit B27. Local reaction, paralysis; recovered.

April 25, 1906. Nos. 4495A, 4495B, and 4495C born.

June 22. Nos. 4495A and 4495C given .007 cubic centimeter toxine No. 7. No reaction third day. Controls died in two days, two hours, and one day, twelve hours.

The results of the test showed a distinct immunity to diphtheria toxine.

June 26. Both of the above, and 4495B, which had received no toxine, were given two cubic centimeters of normal horse serum. All three died in less than forty-five minutes.

FAMILY No. 2.—Mother guinea-pig No. 4: treatment as follows:

May 25, 1905. Given .15 cubic centimeter toxine No. 7 (L + .21) + 1 immunity unit Ehrlich 15.IV.05.

May 29. Same treatment.

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June 9. .18 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

June 16. .19 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

June 26. .19 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

July 12. .19 cubic centimeter toxine No. 7 + 1 immunity unit Ehrlich 15.IV.05.

May 21, 1906. Three young born, 4E, 4F, and 4G.

June 22, 1906. Guinea-pigs Nos. 4E, 4F received 8½ times the MLD. Both showed slight induration on the third day; recovered.

June 26. Guinea-pigs Nos. 4E, 4F, and 4G were given two cubic centimeters normal horse serum. All three died in less than forty-five minutes.

FAMILY No. 3.— Mother guinea-pig No. 8: treatment as follows:

May 25, 1905.	Given	250	units	antitoxine.
June 9,	"	600	"	"
June 16,	"	900	"	"
June 26,	"	1,000	"	"
July 12,	"	1,500	"	"

May 20, 1906. Three young born, 8G, 8H, and 8I.

June 22, 1906. Guinea-pig No. 8H given 8½ times the MLD. Showed slight induration on the third day; recovered.

June 26. Guinea-pigs Nos. 8H and 8I given two cubic centimeters normal horse serum. Both died in less than forty-five minutes.

Four control pigs of about the same age and weight, born of untreated mothers, received four cubic centimeters — twice the amount given the test pigs — of normal horse serum. None of them showed any inconvenience as a result of the injection.

It will be readily seen from the above that a mother can transmit to the same young two properties, viz.: immunity to diphtheria toxine, and hypersusceptibility to horse serum.

It is, therefore, very manifest that female guinea-pigs that have received the toxine-antitoxin mixtures should not be used as breeders by those engaged in work with diphtheria antitoxin. On account of the transmitted immunity misleading results in the testing of antitoxin and toxine will be had. On account of the hypersusceptibility to the injection of horse serum pigs derived from such a source, when used to determine the presence of bacterial or toxic impurities, may die, not from the presence of such impurities, but on account of a hypersusceptibility to horse serum transmitted from the mother.

MULTIPLE MYELOMA WITH INVOLVEMENT OF THE ORBIT.*

ALEX. QUACKENBOSS, A.M., M.D.

(PATHOLOGICAL EXAMINATION BY F. H. VERHOEFF, A.M., M.D.)

A. S., male, fifty-eight years of age, born in Canada, was admitted to the Massachusetts Charitable Eye and Ear Infirmary on Nov. 13, 1905. His parents lived to an old age, one brother died of a "tumor," another brother is living at the age of fifty, but in poor health. Mr. S. has always been well up to the present illness; two years ago had nasal polypus removed. Six months before entrance to the hospital he complained of seeing double, and shortly after noticed that the left eye was more prominent than the right, a condition that has gradually increased up to the present time. Two months ago noticed swelling at the upper part of the sternum. Has been hoarse for the past few months.

Physical Examination: Fairly well developed and nourished anemic. Over sterno-clavicular articulation is a smooth rounded tumor 6 1/2 × 8 1/2 centimeters in size. Lungs normal; heart and arteries show signs of arteriosclerosis. Abdomen negative.

Eyes, right, vision normal, few fine opacities in lens, fundus normal. Left, vision 1/10, not improved with glass, fine diffuse opacities in lens, optic neuritis. At the upper and outer half of the orbit, is felt a hard bone-like mass, in shape and size resembling somewhat the lachrymal gland, firmly attached to the orbit wall. Toward the nasal side another tumor is felt, but more elastic to the touch and situated deeper in the orbit.

Naso-pharynx negative.

Examination of the blood showed nothing remarkable. The first routine examination of the urine, made upon entrance to the Infirmary, showed the presence of albumen.

An exploratory operation was advised, and on November 20 an incision was made along the upper, outer edge of the orbit, and a hard cartilaginous tumor was found, firmly attached to the bone. In attempting to remove this tumor, considerable force was employed, and it collapsed, the contents, a gelatinous material, escaping. Part of the bony wall or shell of the tumor, with quite an amount of its contents, were secured for pathological examination. At the same time a piece from the growth over the sternum was removed.

Following the operation on the eye there was marked swelling of the upper lid and exophthalmos, the eye being pushed forward so that it was impossible to keep the cornea protected, and a superficial ulceration resulted. This swelling gradually disappeared, the eye fell back into place, and the ulcer on the cornea healed. The wound along the orbit healed nicely.

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* Read before the American Ophthalmological Society, New York, June, 1906.

After Dr. Verhoeff examined the specimens sent to the pathological laboratory, he suggested the examination of the urine for albumose, and it was found to be present.

Dr. L. J. Henderson of the department of biological chemistry, Harvard University, to whom a specimen of the urine was submitted, reported as follows: "The urine which you ask about contained on my first examination of it at the Eye and Ear Infirmary, a considerable quantity of a proteid substance which precipitated, on warming to fifty or sixty degrees, redissolved on boiling, and reprecipitated on cooling. The later specimen of urine, which I received here, contains a trace only of this proteid, so that a careful characterization of it is impossible with the material on hand. However, I think that it may be regarded as the 'Bence Jones'' body or an allied substance."

The use of Coley's serum was advised, but after the eye quieted down the patient wished to go home.

Mr. S. was in the hospital five weeks, during this time there was a gradual loss of strength, but at no time did he complain of pain. There was more or less digestive disturbance, and several attacks of nose bleed.

Through the kindness of Dr. A. J. Gallison, of Franklin, Mass., I was able to follow the case. He writes that S. failed steadily after leaving the Infirmary, the growths in the orbit and sternum increased, and in addition four new growths appeared over the right thigh. There was considerable hemorrhage from the nose, towards the end, but the patient kept up and about until twenty-four hours before death, which occurred Jan. 16, 1906. No autopsy.

Duration of disease: From the time he first noticed trouble with his eye, eight months.

The etiology of this disease is obscure, the cases reported have been mostly among the laboring classes. Heredity and trauma have been suggested as possible causes. By some it has been considered an infective process. Syphilis has no bearing. It is more common in males than females, and usually occurs in the middle or later part of life.

Its characteristics are pathological changes in the bone marrow, found in connection with a peculiar proteid body (Bence Jones') in the urine. The disease seems to be closely allied to sarcoma, but differs from it, in that several parts of the body are attacked at the same time, and that there is no tendency to invade other organs by metastasis. It is confined to the skeleton; the ribs, sternum, and vertebræ being a favorite seat; the pelvis, skull, and long bones may be involved; few, if any, cases affecting the orbit have been reported. The new growth is soft and gelatinous, and may be confined to the interior of the bone, or break through and form a tumor of considerable size.

The symptoms vary greatly according to the location. In some cases pain is the predominant feature, while in others it is entirely absent; marked deformity of the skeleton is reported in certain cases, in others nothing abnormal is to be seen. Spontaneous fracture of the long bones may occur. Bone symptoms may be slight or absent, and the nervous system show signs of marked disturbance. Anemia and increasing weakness are constant.

Urine: Most cases of multiple myeloma show the presence of albumose in the urine, the amount present in any given case is not constant, and may vary during the course of the disease. Many consider as albumose all proteids in the urine which dissolve on heating, to reappear on cooling, in the presence of certain reagents. Albumosuria shows that a destructive process is going on in some part of the body, and may be found in multiple myeloma, and in some forms or stages of tuberculosis, lobar-pneumonia, septicemia, pyemia, sarcoma, and carcinoma. Aside from the presence of albumose, the urine shows no special characteristics: the amount may be normal, increased or diminished. The condition is not necessarily associated with pathological changes in the kidneys; some examinations have shown that the kidneys were normal, while in others interstitial nephritis was found to be present.

Prognosis is bad, the disease usually ends fatally inside of two years: one case is reported to have lived seven years.

Treatment is of little avail, arsenic, X-rays, and Coley's serum have been advised.

PATHOLOGICAL EXAMINATION. — The material submitted for examination consists of tissue removed from the orbital growth, some of which contains bone broken from the margin of the orbit, and also tissue from the growth over the sternum. Macroscopically the tissue from both situations is soft, translucent, and slightly yellow in color, somewhat resembling adipose tissue, and its cut surface is more or less distinctly lobulated. Fixation in Zenker's fluid.

On microscopic examination, the tumor presents much the same appearance throughout, having in a general way the structure of a small round cell sarcoma. The individual cells of the parenchyma are loosely packed together in a uniform mass through which run septa of connective tissue carrying the blood vessels. At the advancing margins of the growth it makes use of the invaded connective tissue for its stroma. The tumor cells are remarkably uniform in size and appearance and when stained by hematoxylin and eosin are indistinguishable from plasma cells. They are round or ovoid in shape, their cytoplasm is strongly basophilic, and their nuclei have the eccentric situation seen in plasma cells. The chromatin in the nuclei shows the arrangement characteristic of plasma and lymphoid cells, presenting coarse granules attached to the thick nuclear membrane. Most of the cells are mononuclear, but occasionally a cell contains two to four nuclei.

Special staining* brings out in each nucleus a perfectly definite nucleolus (Fig. 1.) which in the alum hematoxylin specimens is masked by the deeply-staining chromatin. In sections stained in Mallory's phosphotungstic acid hematoxylin † each cell shows in its cytoplasm, contiguous with the nucleus, an ill-defined round area paler than the rest of the cytoplasm which contains in its center two deeply-staining blue dots (centrosomes), (Fig. 2). Sometimes there seems to be one dot only, but careful focussing usually shows that this is due to one dot hiding the other from view. Where two nuclei occur in a cell, four dots are usually to be made out, although only one pale body is present (Fig. 3). Mitotic figures are abundant in the tumor of the sternum, but are not found in the orbital growth. Shrunken nuclei with deeply-staining chromatin are frequently seen, but these are no doubt simply degenerated. The cytoplasm of some of the cells with degenerated nuclei has taken on a hyaline appearance and stains intensely in eosin, so that the cells closely resemble nucleated red blood corpuscles (Fig. 4). Hyaline globules of the same size as these cells, and evidently of the same nature, from which the nuclei have disappeared, are also not infrequently encountered. A much more common form of degeneration, however, if such it is, consists in the occurrence of numerous minute vacuoles at the periphery of the cytoplasm, giving the cell a frayed out appearance. Often this change is so marked that by a coalescence of the vacuoles a clear peripheral zone is produced which separates the cytoplasm from the cell membrane.

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* Celloidin sections are stained for twenty-four hours in a two per cent solution of eosin in eighty per cent alcohol. Water, potassium permanganate 1:300, thirty seconds. Water, oxalic acid 1:1,000 until color of eosin returns, one to three minutes. Water, alcohol, etc.

† Before staining in this fluid the sections should be treated as follows: Potassium permanganate 1:300, fifteen minutes. Water, oxalic acid 1:1,000 until yellow color is removed. This differentiates both the centrosomes and the fibroglia fibrils.

The stroma of the tumor consists of delicate fibrillar connective tissue, which marks it off into more or less distinct alveoli, and of reticulum. The latter cannot be made out everywhere, owing, probably, to the rapid growth of the tumor. It is brought out best by Mallory's connective tissue stain. Accompanying the stroma there are an unusual number of Mallory's fibroglia fibrils. These are apt to be extremely long and can often be traced into relationship with flat cells having elongated nuclei in the manner described by Mallory for normal tissues. The fibroglia fibrils are best seen in sections stained in phosphotungstic acid hematoxylin, but are also well brought out by the acid fuchsin in the connective tissue stain. The fibrils are also found in great numbers running between the individual tumor cells, although no doubt in every case they originate in the connective tissue stroma.

The growth is well supplied with blood vessels. Some of the smaller vessels have very thin walls and are immediately surrounded by the tumor cells, as is usually the case in sarcomata. There are no areas of complete necrosis or hemorrhage and the tumor is nowhere infiltrated with chronic inflammatory cells. There are also no phagocytic epithelioid cells such as are seen in typical lymphoid tissue.

Except for the slight but distinct difference in the character of the cells, the appearance presented at the margin of the growth resembles that of a chronic inflammatory infiltration. The tissue removed from the margin of the orbit shows extensive invasion of the medullary spaces by the tumor, but the bony lamellæ are not directly attacked.

Diagnosis: Multiple myeloma involving sternum and orbital wall.

The character of the tumor cells in this case and the occurrence of nodules in two situations leave no doubt that the growths belong to the type of tumors classed as multiple myelomata. In fact, the morphology of the cells is alone sufficient for the diagnosis since so far as is known such cells are peculiar to these tumors. The exact nature of the cells has not yet been definitely determined. All that is certainly known about them is that they arise in the bone marrow. Most observers* have regarded them as allied to myelocytes though attempts to demonstrate neutrophile granules in them have practically always failed. Wright has advanced the view, recently supported by Hoffman, that the cells are plasma cells. This view seems to accord best with the findings in the present case. The only difference that could be made out here between the tumor cells and plasma cells was the presence of fully differentiated nucleoli in the former, and this perhaps could be accounted for by the rapid proliferation of the tumor cells. The finding by special staining of centrosomes in the cells of this case is also in favor of the plasma cell theory. Plasma cells found in chronic inflammatory conditions, which were subjected to the same staining method, showed centrosomes having exactly the same situation and appearance as those in the tumor. However, it must be noted that this method shows similar centrosomes in a variety of other cells.

Ribbert has recently reported a case in which he believed he was able to demonstrate a transition of tumor cells into nucleated red blood corpuscles. He therefore took the view that the tumor cells in his case were the anlages of red blood corpuscles. In the present case cells similar to nucleated red blood corpuscles were also found, but they were regarded as degenerated plasma cells. Such cells are often formed by the degeneration of plasma cells in many pathological conditions, hence their occurrence in the tumors would seem to support the view that the cells of the latter are plasma cells rather than that they are nucleated red blood corpuscles. It does not seem impossible, however, that red blood corpuscles are normally formed by a degeneration of this kind and that the similarly degenerated plasma cells found in chronic inflammatory conditions really represent atypical nucleated or even non-nucleated red blood corpuscles. An attempt to reconcile the different views in regard to the nature of the cells of multiple myelomata has been made by Scheele and Herxheimer, who assume that the cells arise from undifferentiated elements of the bone marrow and thus may at times become differentiated into myelocytes, plasma cells or erythroblasts.

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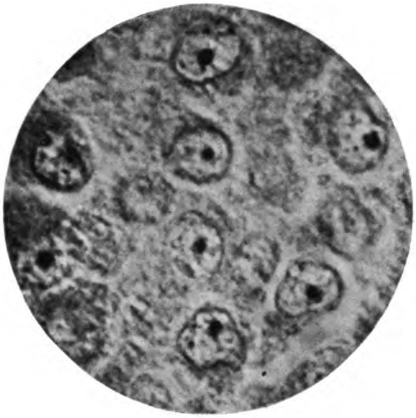
* The complete literature on multiple myeloma has been collected by Menne, Zür Kenntnis der Myelomzellen. Virchow's Archives, 1906, clxxxiii, 115.

DESCRIPTION OF PLATE.

1. Photo x 1500, showing nucleoli in tumor cells. Special eosin stain.

2, 3. Tumor cells showing centrosomes. Phosphotungstic acid hematoxylin. Zeis obj. 1/12, oc. 4.

4. Degenerated tumor cell simulating nucleated red blood corpuscle. Hematoxylin and eosin. Zeis obj. 1/12, oc. 4.



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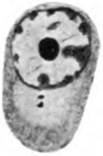

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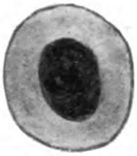

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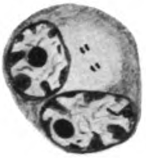

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Quackenboss and Verhoeff.

Multiple Myeloma.

THE VIABILITY OF BACILLUS TYPHOSUS UNDER VARIOUS CONDITIONS.*

JOHN M. WHEELER, M.D.

INTRODUCTION.

It is generally conceded that the disease most to be dreaded from drinking "impure water" is typhoid fever. It is also conceded that the causative agent of this disease is the organism bacillus typhosus. Therefore, a great many observers have turned their attention toward this organism, and much experimental work has been done with a view to ascertaining its behavior in water under different conditions. Yet the statements in our text-books in regard to this are for the most part general, and notably contradictory. For example, Osler[1] says: "In sterile water the bacilli retain their vitality for weeks, but under ordinary conditions in competition with other saprophytes they disappear within fourteen days. Whether an increase in water can occur is not finally settled, but it probably may take place to some extent at first. . . . In ice they may live as long as eighteen weeks, though a majority die in two weeks."

Muir and Ritchie[2] in their text-book on bacteriology say: "Typhoid bacilli kept in distilled or ordinary tap water have usually been found to be dead after three weeks."

Stengel[3] is responsible for the statement that "the typhoid bacillus is peculiarly resistant, and may thrive upon clothing, in soil and in water for a long time. Cold has no effect, the germ being virulent after freezing and thawing several times."

But Prescott and Winslow[4] say that "in ice or cool water forty per cent perish in three hours, and ninety-eight per cent in two weeks," and[5] "they may persist in sterilized water for upwards of two months, and in unsterilized water from three days to several weeks."

It is evident, then, that a number of elements enter into the problem of the behavior of the typhoid bacillus in water. Probably the more important factors are the conditions of

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*Received for publication July 3, 1906.

the organism as to strain, or variety, and vitality, the character of the water and the degree of light to which the organism is subjected.

The strain of the organism is probably an unimportant factor. For when series of experiments have been carried out with organisms from different sources, the results with one strain of the organism have been almost identical with those done with others. For example, in a series of experiments on the longevity of the typhoid bacillus carried on by Jordan, Russell and Zeit[6] organisms from three different sources were used, and no difference in results obtained could be ascribed to a difference in behavior of the strains of organisms used. In fact, comparative work done with the typhoid bacillus and allied forms, such as the paratyphoid and colon bacillus, has shown a marked similarity of behavior on the part of these organisms. It is probable, however, that the length and character of artificial cultivation has some influence on the longevity of the typhoid bacillus in water.

The character of the water containing the organisms is an important factor, and involves a number of problems but imperfectly understood. The more important of the influences under this heading are probably: *a*, Inorganic matter and its character; *b*, amount of oxygen in the water; *c*, organic pollution and its character; *d*, temperature of water; *e*, if sterile, the mode of sterilization; *f*, presence of organisms other than B. typhosus.

From the work done, the effect which light has on bacteria in water seems to be in proportion to the degree of light to which the water is exposed.

It is possible that other factors, such as the degree of motion of the water and size of the body of water, are of considerable importance in determining the viability of the typhoid bacillus in water.

The original experiments recorded below were all carried out at the Vermont State Laboratory of Hygiene at Burlington in the years 1904, 1905, and 1906.

Unless otherwise specified, a stock culture of B. typhosus used in routine work at the laboratory was employed in the experiments. This strain was obtained from Dr. Park, of New York, and has been under artificial cultivation for a number of years. It is known as the Ehrlich strain. In all cases broth cultures (twelve to twenty-four hours) were used for inoculation.

1. Inorganic matter in the water. — Dr. Moore,[7] Professor Kraemer[8] and others have pointed out that minute quantities of copper in solution in water have a decidedly detrimental effect on the typhoid bacillus. More recently Clark and Gage[9] have demonstrated that the effect of several other metals is fully as marked as that of copper. This is suggestive that inorganic metallic constituents may play an important rôle in influencing the life history of B. typhosus in natural waters.

2. Oxygen in the water. — Recently, Whipple and Mayer,[10] by a series of comparative experiments, have demonstrated that the longevity of the typhoid bacillus is much greater in water to which oxygen is given access than in water from which this gas has been excluded. It is possible that this factor is a much more potent one than has been realized by most observers.

Experiment No. 1. — This experiment was done with a view to determining the effect of the kind of water, moderate degrees of temperature, and diffuse daylight upon the typhoid bacillus in water.

Pint bottles of plain glass were used as receptacles. Into each were put two hundred and fifty cubic centimeters of water as follows: four bottles, distilled (single) water; four bottles, Burlington tap water; four bottles, polluted well water.

The analysis of Burlington tap water and the polluted well water used is given in the appendix.

The still used is one lined with block tin.

Three kinds of water: Single distilled, tap, polluted well.

Three temperatures: Refrigerator, 10°–12° C.; room, 20°–22° C.; body, 37° C.

Two degrees of light: Dark, diffused daylight.

The bottles of water were sterilized in the autoclave. Then each bottle was inoculated with one drop of a twenty-hour bouillon culture of B. typhosus.

The bottles were divided into four groups of three, the groups containing one bottle each of distilled, tap, and well water.

One group was placed in the refrigerator (10°–12° C.); another group in a room temperature incubator (20°–22° C.); another on a shelf about three feet from and to one side of a north window, which at no time admitted sunlight; and still another group of three in a body temperature incubator (37° C.).

Agar plates were poured immediately after inoculation and every second day thereafter. The colonies were counted after two days' incubation in the body temperature incubator.

To detect possible contamination, colonies were frequently fished from the plates and incubated at 37° C. in bouillon. Hanging drops of the broth culture were examined, and the broth tested for the Widal reaction.

So large a proportion of the bottles of water were contaminated in the first trial that a tabulated report is not given except in the case of the water kept in the ice-chest.

This record is found in the second column of the tabulations under each kind of water. (Column b.)

The difference in the behavior of the typhoid bacillus in the well water at room temperature from that in the distilled water and tap water was so marked that it was thought possibly to be accidental. So another bottle was prepared as before and set in the room temperature incubator. The counts from this bottle are found in column e of the tabulated sheet.

The numerical results are as tabulated below, the figures in each column representing the number of organisms per cubic centimeter of the water.

DISTILLED WATER.

Days.	Dark.					Light.
	Ice-chest, 10°–12° C.		Body Temp. 37° C.	Room Temp. 20°–22° C.		
	a.	*b.*	*c.*	*d.*	*e.*	*f.*
1.....	14,450	350	8,300	2,050	....	20,300
3.....	3,000	50	400	....	....	300
5.....	400	50	....	3,500	....	50
7.....	125	20	0	4,050	....	50
9.....	....	30	0	1,450		
11.....	75	30	0	1,790	....	10
13.....	25	0	2	1,100	....	2
15.....	0	0	1	550	....	0
17.....	1	0	0	500	....	0
19.....	0	....	0	....	....	0
21.....	0	....	0	85	....	0
23.....	0	....	....	10		
25.....	....	....	....	7		
27.....	....	....	....	2		
29.....	....	....	....	0		
31.....	....	....	....	1		
33.....	....	....	....	1		
35.....	....	....	....	0		
37.....	....	....	....	1 in 2 cc.		
39.....	....	....	....	0		
41.....	....	....	....	0		

BURLINGTON TAP WATER.

Days.	Dark.					Light.
	Ice-chest, 10°–12° C.		Body Temp. 37° C.	Room Temp. 20°–22° C.		
	a.	*b.*	*c.*	*d.*	*e.*	*f.*
1	500	200	5,700	5,600	....	11,950
3	2,000	100	7,000	200	....	100
5	....	150	400	50	....	0
7	2,375	20	150	50	....	0
9	1,250	30	....	25	....	10
11	....	10	10	30	....	0
13	....	3	2	18	....	0
15	20	0	3	18	....	1
17	4	0	1	22	....	0
19	4	0	0	21	....	0
21	3	0	0	29	....	0
23	0	....	....	24		
25	0	....	....	8		
27	....	....	....	3		
29	....	....	....	3		
31	....	....	....	1		
33	....	....	....	1		
35	....	....	....	1		
37	....	....	....	2		
39	....	....	....	0		
41	....	....	....	0		
43	....	....	....	1 in 2 cc.		
45	....	....	....	0		
47	....	....	....	0		
49	....	....	....	0		

POLLUTED WELL WATER.

Days.	Dark.					Light.
	Ice-chest, 10°–12° C.		Body Temp. 37° C.	Room Temp. 20°–22° C.		
	a.	*b.*	*c.*	*d.*	*e.*	*f.*
1	16,150	150	5,700	9,150	....	5,750
3	6,600	450	7,000	12,000	120,000	3,000
5	3,200	140	400	39,050	340,000	1,500
7	3,500	40	150	112,500	504,000	50
9	3,700	30	....	252,000	376,800	20
11	3,200	50	10	360,000	240,800	10
13	200	90	2	373,200	202,400	2
15	80	4	3	408,000	269,000	1
17	85	2	1	424,000	252,000	0
19	52	2	0	422,000	214,000	0
21	27	2	0	202,400	178,200	0
23	17	3	0	192,600	104,400	0
25	8	2	....	16,000	109,200	
27	9	2	....	137,000	68,000	
29	3	1	....	97,800		
31	0	0	....	93,600	59,600	
33	0	0	....	....	58,000	
35	1	0	....	68,000	47,600	
37	1	0	....	48,400	49,500	
39	0	....	....	32,000		
41	0	....	....	22,100	22,000	
43	....	....	....	17,400	26,600	
45	....	....	....	10,600	13,750	
47	....	....	....	50	2,050	

POLLUTED WELL WATER. — *Continued.*

Days.	**Dark.**					**Light.**
	Ice-chest, 10°–12° C.		**Body Temp. 37° C.**	**Room Temp. 20°–22° C.**		
	a.	*b.*	*c.*	*d.*	*e.*	*f.*
49....	....	....	....	5,050	4,250	
51....	....	....	....	3,050	2,350	
53....	....	....	....	1,450	1,090	
55....	....	....	....	790	520	
57....	....	....	....	600	630	
59....	....	....	....	....	70	
61....	....	....	....	200	contaminated	
63....	....	....	....	50		
65....	....	....	....	41		
67....	....	....	....	19		
69....	....	....	....	10		
71....	....	....	....	4		
73....	....	....	....	0		
75....	....	....	....	0		
77....	....	....	....	1 in 2 cc.		
79....	....	....	....	1 in 2 cc.		
81....	....	....	....	0		
83....	....	....	....	0		
85....	....	....	....	0		

The maximum length of life under the different conditions is summarized in the table below:

	Dark.			Light.
	10°–12° C. Ice-chest.	37° C. Body Temp.	20°–22° C. Room Temp.	
Distilled water	17 days.	15 days.	37 days.	13 days.
Tap water	21 days.	17 days.	43 days.	15 days.
Well water	37 days.	17 days.	79 days.	15 days.

It is evident from examination of these tabulated results that in sterilized water the presence of organic matter is favorable to the multiplication and longevity of B. typhosus; that diffused daylight acts unfavorably; that room temperature is more favorable than body temperature or ice-chest temperature. The optimum conditions, then, appear to be a temperature of about 20°–22° C., the presence of considerable organic pollution and the exclusion of light. It is seen that under these conditions considerable increase in numbers takes place for about two weeks, that the decline which follows is gradual, and the persistence of the organism is much longer than when these conditions are not operative.

3. Light. — It is generally agreed that sunlight is detrimental to the typhoid bacillus.

Clark and Gage[11] found that when in water spread out in a thin layer in the sunlight they were destroyed in one hour or less, and when exposed in bottles sterilization was complete in five hours.

Apparently, little work has been done to show the effect of diffused daylight on the organisms in water.

Büchner[12] calls attention to the germicidal action of sunlight, and to the less powerful action of diffused daylight. After a series of experiments not related in detail, he concluded that sunlight is a more potent factor in reducing the number of bacteria in natural bodies of water than sedimentation and other factors. And to make a practical application he suggested running sewage into large, flat reservoirs for disinfection.

Procacci[13] exposed water in deep cylinders to the nearly vertical rays of the sun, and found that after three hours' exposure, the water in the cylinders to one foot in depth was nearly sterile, while at a depth of two feet the typhoid bacilli were unaffected.

The results of my experiments show diffuse daylight to have a detrimental action on B. typhosus in the three varieties of water employed. But its action is most manifest in the case of the well water, in which typhoid organisms live for seventy-nine days in the dark, as against fifteen days in the light. It is also noticeable that marked reproduction takes place in the dark, whereas in the light there is a gradual decline in numbers from the first.

The detrimental effect of diffuse daylight is confirmed in a later experiment (Experiment No. 8, page 289).

Further experiments along this line would be of value. The depth at which the germicidal action of light is active could be determined more or less satisfactorily by sinking sealed typhoid inoculated bottles of water at different depths in a body of water exposed to light and making comparative counts at regular intervals. It seems probable that the germicidal action of diffused light has been under-estimated in considering the longevity of the typhoid bacillus in water.

4. Organic pollution. — The question as to whether the viability of B. typhosus is or is not favored by the presence of organic pollution in the water is one which has received different answers.

Osler[14] says: "Filth, bad sewers or cesspools . . . furnish the conditions suitable for the preservation of the bacillus, and possibly for its propagation."

On the other hand Jordan, Russell and Zeit[15] agree "that when typhoid bacilli are introduced into unsterilized water containing little organic matter, their longevity is more prolonged than in water charged with considerable organic matter."

If these latter observers are correct in their views, it may be that the amount of oxygen present in the water is an influential factor. For we should expect water containing a large amount of decomposing organic material to have a smaller oxygen content than that containing little organic matter. Reference is made under another heading (page 271) to the fact that the typhoid bacillus thrives better in water containing oxygen than in that deprived of it.

The degree to which the presence of organic matter favored the viability of B. typhosus in sterilized water in my experiments was considerable at all temperatures, but most marked at room temperature. In the room temperature incubator, the organisms not only remained present for a much longer period of time in the polluted well water, but showed a far greater power of reproduction in the well water than in either distilled or Burlington tap water. But it should be borne in mind that at least one factor which is generally recognized to be of importance is eliminated in the use of sterilized water. This is the antagonism to the typhoid bacillus exerted by saprophytes which exist in such great numbers in water high in organic pollution.

5. Temperature, moderate degree. — In bouillon the optimum temperature for growth of the typhoid bacillus is about that of the body (37° C.). This is suggestive that this temperature of water might be the most favorable to the typhoid organism. But such does not seem to be the case.

Clark and Gage [16] found the maximum temperature in water which B. typhosus can endure to be about 80° C. for five minutes exposure, and they found body temperature to be less favorable to the typhoid bacillus than room temperature.

Konradi and Bolton [17] both found that the typhoid bacillus lived longer at room temperature than at body temperature.

Reference to the tabulated results of my experiments shows the length of life and power of reproduction to be greater at room temperature than at ice-chest or body temperature in distilled, tap, and polluted well sterilized water.

A temperature of about 20–22° C. was found to be optimum for B. typhosus.

The effect of temperature below the freezing point is taken up under a separate heading.

Low degrees of temperature. — In 1887 Prudden[18] carried on a series of experiments in connection with his investigation of the ice supply of New York City. He put into a series of sterile tubes five cubic centimeters of water which he had sterilized and seeded with B. typhosus, and then froze the water at 14° to 30° F. At the end of one hundred and three days he found large numbers of the organisms alive. At the end of this period the experiment was discontinued. As a result of his investigation, Prudden concluded that ice frozen from sewage contaminated water was a real source of danger as to typhoid infection.

Recently Zeit[19] performed an experiment similar to that by which Prudden obtained his results. He used the same amount of water (filtered Chicago river water) in test-tubes, excluded light, and admitted air by the use of cotton plugs, as did Prudden. Yet, on melting the ice, and plating in agar all the water in the tubes, he found no typhoid organisms present after the first day.

Clark and Gage[20] report a series of experiments on the viability of B. typhosus in ice, and also in water at about 33° F. They conclude that most organisms are destroyed quite rapidly by the effect of cold either in fluid culture or when frozen in ice. Clark[21] points out that in the process of freezing the organisms are expelled from the water frozen into the water below, and claims to have established it "by determination of the numbers of bacteria in the ice, and in the water under the ice, and by chemical analyses which showed that the greater portion of insoluble organic matter is expelled from ice during natural freezing."[22] But, they (Clark and Gage) say that when caught in ice the germs will live "for a considerable length of time." But in their experiments the morphological and cultural characteristics were so different from the original type that these organisms were unrecognizable. Nor could they be reverted to the original type by extended cultivation.

In the winter of 1904-1905 B. H. Stone[23] carried on experiments at the Vermont State Laboratory of Hygiene to show the effect of low temperature on typhoid infected water. In these experiments a sixteen quart tin pail, with a cover, and with sides sloping so as to make its diameter at the top about two inches greater than at the bottom, was used. This pail was nearly filled with tap water, sterilized and then inoculated with typhoid bacilli when the water had cooled. The pail with its contents was then packed in sawdust so that freezing would take place mostly from the surface, as occurs in nature. The temperature of this water was then lowered to near the freezing point, and agar plates made from the surface. About five cubic centimeters of the seeded water was then dipped from the surface with a sterile tin capsule about two inches in diameter. This capsule with its contents was floated upon the surface of the water in the pail. The pail was then covered and placed outside in zero (F.) weather.

After twelve hours the ice had acquired a thickness of one-eighth of an inch, and the water in the capsule was entirely frozen. Pieces of ice were removed from the surface and melted in sterile plates, and the ice in the capsule was melted. Relative counts from agar plates which were poured showed a reduction from 6,000 organisms per cubic centimeter on the surface before freezing to twenty per cubic centimeter in the ice outside the capsule and sixty per cubic centimeter within the capsule.

Again surface water was dipped into the capsule, and the pail of water with the capsule floating on the surface was exposed to the cold. At the end of twenty-four hours or thirty-six hours from inoculation one-half inch of ice had formed on the surface, and plating showed the ice outside the capsule to be sterile, and the number in the ice within the capsule was further reduced to thirty-five per cubic centimeter.

On repetition similar results were obtained. It was also found that after thirty-six hours' exposure the unfrozen water at the bottom of the pail was sterile. The water in the pail finally was allowed to freeze solid and was found to be sterile after twelve days' exposure.

Dr. Stone calls attention to the fact that the conditions of these experiments are somewhat artificial, as no water which contains typhoid bacilli would ever be found in nature free from other organisms, "but it can hardly be conceived that the presence of other bacteria would render the typhoid germs more resistant. In fact the effect would probably be the reverse."

FURTHER EXPERIMENTS. — In the winter of 1905-1906 a series of experiments was carried out by the author with the use of methods similar to those which Dr. Stone had employed the year before. These experiments were made with a view to getting more information on the following points: 1, The effect of freezing the typhoid germs in water. 2, The effect of cold (about 0° C.) on the organisms in the water under the ice. 3, The matter of expulsion of the organisms into the water below in the process of freezing.

Experiment No. 2. — In this experiment the tin pail which Dr. Stone had used the previous winter was employed. It will be noted that Dr. Stone's experiments did not seem to substantiate the conclusions of Clark and Gage relative to the expulsion of the typhoid bacillus in the process of freezing, inasmuch as there was no marked difference in the bactericidal effect of freezing, whether within the capsule (where expulsion was impossible) or outside the capsule. However, it occurred to the writer that the metal of the capsule itself might have some bactericidal influence on the organisms in the small amount of water contained therein.

So in this experiment, as in the rest of this series, porcelain capsules were substituted for tin and a little more water than before was dipped from the surface. In this experiment, as in all others where agar plates were used for making counts, the plates were incubated for forty-eight hours or longer.

Into the pail were poured seven liters of Burlington tap water. Porcelain capsules were floated on the surface, and the outfit (with cover on) was sterilized. When the water was cool it was inoculated with five cubic centimeters of a twelve-hour broth culture of B. typhosus. The water was stirred with a sterile pipette, and plates were made from different depths. The pail was then packed in sawdust and covered with a sterile cloth and exposed to a temperature of about 0° F.

In twelve hours about one-half inch of ice had formed on the surface, and a thin layer had formed about two inches down the side of the pail. The water in the capsules was entirely frozen. The ice in one of the capsules was allowed to melt at body temperature after placing upon it a sterile glass cover. Pieces of the surface ice outside the capsule were dipped in boiling water with sterile forceps to sterilize the surface; then after a portion was melted off the balance was allowed to melt in sterile dishes. Duplicate plates were poured from the melted ice, using one cubic centimeter in each, and the average counts before setting out and after twelve hours' exposure follow.

	Before Exposure.	After 12 Hours.
Water	3,600	
Water directly under ice	....	0
Water at bottom under ice	....	0
Ice in capsule	....	0
Ice outside capsule	....	0

Experiment No. 3. — Total destruction of the bacilli in so short a time in the previous experiment caused suspicion that some force other than that of cold was operative. It seemed possible that the metal of the pail might have some effect here. So a pail enamelled with "water glass" (sodium silicate) was obtained, with diameters of ten inches at the top and seven at the bottom, and a glass cover was adjusted. This was substituted for the tin pail.

It seemed advisable to try another strain of the typhoid bacillus. So in this experiment an organism was used which was obtained from the botanical department of the University of Vermont, and which in turn was obtained by them from Dr. Moore of Cornell Veterinary College.

Five liters of tap water were used, and porcelain capsules were floated as before. Temperature about 5° F. In twelve hours the water was frozen solid. Plates were poured from the ice in the capsule, from the ice on the surface outside the capsule, and from ice on the side and bottom of the cake.

Average counts were as follows: Water before exposure, 58,600. Twelve hours after exposure: Ice, capsule, 209; ice, outside on surface, 624; ice, side and bottom of cake, 750.

Colonies were fished from all the plates and incubated in broth at body temperature. In twelve hours clouds appeared in broth. Hanging drops showed organisms typical in appearance but having almost no motility. After transfer into sterile broth and twenty-four hours' incubation a large proportion of the organisms showed motility and responded to the Widal agglutination test.

The ice was allowed to melt slowly, and when it was entirely melted, plates were poured from the water at the top and bottom, one and two cubic centimeters per plate. No colonies developed on the plates. One and two cubic centimeter samples were pipetted into broth, however, and typhoid organisms developed. So apparently organisms will grow in broth where they will not form colonies in agar plates.

In considering these results it should be noted that the enamel lining of the pail was broken in the freezing process, and the water, on melting, was exposed to the metal. This may have a bearing on the results obtained in this experiment.

On comparing the results of this last experiment with those of Experiment No. 2 and those which Dr. Stone obtained, it is evident that either the Moore strain is more resistant to the freezing process than the Ehrlich strain which was used in the other experiments, or else the change in the vessel in which the water was contained was an influential factor. To determine whether or not the strain of organisms was the responsible factor, in Experiment No. 4 as in all future experiments, the Ehrlich strain was used.

Experiment No. 4. — The cracked pail was replaced by a new one of the same character. Temperature, about 20° F.; amount of water, five liters; preparation as in last experiment.

After twelve hours' exposure the ice in the capsule was not frozen quite solid. About one-half inch of ice had formed on the surface outside the capsule. Counts were made at this time.

After this the temperature rose a little above the freezing point, and remained above for about twenty-four hours. It then went a little below freezing. In forty hours from the time of the first exposure, or twenty-eight hours from the time the first counts were made, the ice was frozen solid in the capsule and about one inch had formed on the surface. So the water under the ice was kept at about freezing point forty hours.

The tabulated results follow:

	Before Exposure.	12 Hours.	40 Hours.
Water	45,000		
Water directly under ice	....	3,960	790
Water deep under ice	....	3,960	850
Ice, capsule	....	3,600*	32
Ice, outside on surface	....	26	160

* Not frozen solid.

By comparing the results of this experiment with those of No. 3 it seems that the Ehrlich strain is fully as resistant as the Moore strain of B. typhosus, and seems to indicate that the metal pail was responsible for some part of the sterilization in Dr. Stone's experiments.

Experiment No. 5. — The mode of procedure was the same as in the previous experiment, only counts were made from the surface and bottom of the inoculated water to determine whether any sedimentation takes place apart from the freezing process.

After inoculation the pail of water was allowed to stand at room temperature for three hours. Average counts made from two plates poured from the water at the top and that at the bottom showed: top, 1,500; bottom, 84,800.

The pail was exposed to a temperature of about 10° F., packed as before. After nine hours' exposure with as little disturbance as possible counts showed: top, 2,800; bottom, 5,150.

In twenty-four hours from inoculation and twelve hours from the last count the ice in the capsule was frozen, and there was a thin layer of ice over the surface. Counts made at this time gave these results: Water directly under the ice, 320; water at bottom under the ice, 6,000; ice in capsule, 17; ice outside capsule, 140.

Counts were made again two days after inoculation. Water directly under the ice, 240; water at bottom under the ice, 1,700; ice in capsule (two cubic centimeters), 0; ice outside capsule, 38.

One cubic centimeter samples from all of the above showed clouds in broth and typical appearance in hanging drop. Growth from ice in the capsule was tested for the Widal reaction and responded.

By pouring sterile broth into a capsule of ice and incubating, typhoid growth was obtained. So that living organisms were shown to occur still in the ice frozen in the capsule.

After three days from inoculation the following counts were yielded:

Water directly under ice, 75; water at bottom under ice, 60; ice in capsule, 0; ice outside capsule, 0.

On incubating in broth the water from all the ice in two capsules it was found on this day to be absolutely sterile.

After five days from inoculation plates were poured from the ice and water, and about thirty cubic centimeters of ice were melted and incubated in broth. Water directly under the ice, 6; water at bottom under the ice, 13; ice plated in agar, 0; ice incubated in broth, 0

For the next two days the temperature was above freezing point most of the time, and most of the ice melted, so the water at the end of these two days was made up of melted ice as well as water which had not frozen.

On the ninth day the ice was entirely melted. Agar plates showed: water near top, one colony to two cubic centimeters of water; water near bottom, 0.

One and two cubic centimeter samples from both near the top and near the bottom added to broth showed typhoid growth.

On the thirteenth day the water was frozen solid. About thirty grams of ice near the center of the cake was melted. Three cubic centimeters were placed in agar and the rest incubated in sterile broth. It was all found sterile.

Five days later more ice was examined in the same way and found sterile.

The results of the four preceding experiments are here summarized in tabulated form to facilitate comparison.

The numbers in the table represent the number of typhoid bacilli per cubic centimeter as estimated from agar plates.

Number of Experiment, etc.	Time from Inoculation.	Water.				Ice.			
		Top.		Bottom.		In Capsule.		Outside Capsule.	
		Agar.	Broth.	Agar.	Broth.	Agar.	Broth.	Agar.	Broth.
Exp II., tin pail, Ehrlich culture.	0 hours.	3,600	. . . .	3,600					
	12 "	0	—	0	—	0	—	0	—
Exp. III., enamelled pail, Moore culture.	0 hours.	58,600	. . . .	58,600					
	12 "	. . . .	. . . .	. . . .	. . . .	209	+	624	+
Exp. IV., enamelled pail, Ehrlich culture.	0 hours.	45,000	. . . .	45,000					
	12 "	3,900	+	3,960	. . . .	3,600*	+	26	+
	40 "	790	+	850	+	32	+	160	+
Exp. V., enamelled pail, Ehrlich culture.	3 hours.	1,500	. . . .	84,800					
	12 "	2,800	. . . .	5,150					
	24 "	320	. . . .	6,000	. . . .	17	+	140	+
	2 days.	240	. . . .	1,700	. . . .	0	+	38	+
	3 "	75	+	60	+	0	—	0	
	5 "	6	+	13	+	. . . .	. . . .	0	—
	7 "	1	+	1	+	. . . .	. . . .	0	—
	9 "	1 in 2 cc.	+	0	+				
	13 "	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	0	—
	18 "	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	0	—

+ = present. — = absent. * = ice not frozen solid.

From the study of the results of these experiments it is evident that the typhoid bacilli in water in the capsule suffered as marked decrease in numbers during the process of freezing and as speedy extermination in ice as occurred outside the capsule. Certain it is that in the freezing process, the typhoid bacilli were not expelled from the water in the capsule into the water below. The theory of expulsion would be a difficult one to prove, and inasmuch as the authors have offered no proof it may be regarded as entirely uncalled for.

Experiment No. 6. — Inasmuch as there was a marked difference between the results obtained when the tin pail was used and those obtained when the enamelled pail with a glass cover was used, it was thought advisable to determine whether or not at room temperature the typhoid organisms had greater viability in the enamelled than in the tin pail. Accordingly five liters of tap water were put into each of the pails and then sterilized. After the water had cooled that in each vessel was inoculated with as nearly as possible the same seeding.

Counts were made from the surface, the bottom, and about midway between (about three inches from.the surface).

Comparative results follow:

Time from Inoculation.	Surface.		3 Inches from Top.		Bottom.	
	Enamelled Pail.	Tin Pail.	Enamelled Pail.	Tin Pail.	Enamelled Pail.	Tin Pail.
6 hours	14,600	2,200	29,000	5,400	28,000	1,400
3 days	23	0	113	0	175	0

It is evident that the tin pail has a decidedly detrimental action on the typhoid organisms in the water in the pail, as in six hours they had undergone a much greater reduction in the tin pail than in the other, and in three days the water was sterile.

It is also evident from this and previous experiments that some sedimentation of the organisms takes place in still water.

Experiment No. 7. Examination of B. coli communis in Ice. — Clark and Gage[24] and many other observers have demonstrated that there is a marked similarity in the viability of B. coli and B. typhosus. Of the two organisms, however, B. coli is the more resistant.

To compare laboratory results with actual findings in nature, it was thought that a fair test would be to examine ice which had formed on the surface of Lake Champlain from water known to be polluted with the colon bacillus. Accordingly, samples of ice were collected from four different places on the lake.

1, Thirty feet from the point where the Burlington sewage empties into the lake; this ice was soft, colored, and contained small particles of sewage material; 2, about four hundred feet south of this point; 3, the slip first south from Maple street; 4, about one hundred feet west of the breakwater out from the foot of Maple street.

In the last three named places the ice was hard and clear. The samples were taken from below the upper layers of the ice, so they would not contain ice formed on the surface of the originally formed ice. This ice had been frozen for about a month.

Samples of water under the ice were gathered in sterile bottles from the same points.

The samples of ice were rinsed in boiling water and allowed to melt in sterile capsules at about body temperature. One, two, and three cubic centimeters from each sample were put into neutral red lactose broth fermentation tubes. These tubes were allowed to remain in the body temperature incubator for forty hours, and no gas or canary yellow color was formed in any of them. So it may be concluded that there were no colon bacilli in six cubic centimeters samples from any of the ice.

One and two cubic centimeters samples from each bottle of water collected were transferred to fermentation tubes. Gas and a yellow color were formed in the tubes from both one and two cubic centimeters samples from the water thirty feet and that four hundred feet from the mouth of the sewer. And the two cubic centimeters sample from water under the ice in the slip first south of Maple street showed gas but no color. The samples from the water outside the breakwater showed neither gas formation nor change of color. In only one tube, however (that containing one cubic centimeter of water from the first point nearest the sewer), was there the typical gas production and canary yellow color. The colon bacillus was evidently overgrown by another vigorous organism, so that it was impossible by the use of acid check solution, broth, and plating, to isolate the colon bacillus from any of the fermentation tubes.

It is beyond doubt, however, that the water from which the ice at the mouth of the Burlington sewage system was formed contained the colon bacillus, as fecal matter was present in both the ice and the water under the ice.

These results, showing the colon bacillus to be absent in ice, are in accord with those obtained by the Massachusetts State Board of Health, as their report in 1900 says: "In not one instance of the still freezing of ordinary polluted water . . . have we been able to find B. coli in the ice formed."

6. Manner of sterilization of water. — Results of experiments done by various observers have led them to believe that there is something in natural waters which exerts a detrimental influence upon the organisms contained therein.

Frost[25] asserts that "there is abundant proof that raw waters are not suited for the development of B. typhosus, but the heating of the same in the process of sterilization renders the water capable of sustaining the organism for a longer time, and under some conditions permits the multiplication of the typhoid germ in them."

An experiment, then, would be interesting which would show whether water sterilized by heat or that sterilized by some other means is more favorable to the typhoid bacillus. Accordingly, the following was carried out:

Experiment No. 8. — Into four one pint glass bottles were put about two hundred and twenty-five cubic centimeters of Burlington tap water, and the bottles were plugged with cotton. The water in these bottles was sterilized in the autoclave. Into each of several sterile bottles of the same size were put two hundred and twenty-five cubic centimeters of tap water which had been passed through a Pasteur-Chamberland porcelain filter, and these bottles were plugged with sterile cotton. Four of these bottles were selected in which by repeated tests the water had been found to be sterile. The water in each bottle was inoculated with one drop of a twenty-four hour broth culture of B. typhosus. After shaking, counts were made at once by plating in agar. Two bottles containing each kind of water were then placed in the room temperature incubator, and two were placed under a bell-jar on a shelf about three feet from a north window, so as to be exposed to diffused daylight.

Every second day water from each bottle was plated in agar in duplicate, and counts were made after forty-eight hours' incubation at 37° C. When the count got very low, samples were incubated in broth, as well as agar. In no case was it found in this experiment that the organisms failed to grow in agar if they grew from samples in broth.

On the third day the agar failed to solidify, and no counts could be made.

This experiment is not yet completed, but the results thus far obtained are tabulated below.

Frequently throughout the experiment colonies from different plates were fished, incubated at body temperature in broth, and examined to make sure that there was no contamination of the water in the bottles.

TEMPERATURE 20°–22° C.

Day.	Dark.				Light.			
	Heated.		Filtered.		Heated.		Filtered.	
1	15,000	13,000	13,200	22,400	13,600	14,400	15,200	11,400
3								
5	1,600	10,000	105,000	100,000	270	130	6,150	28,000
7	750	24,450	336,000	343,200	87	42	17,100	23,800
9	450	96,000	484,800	840,000	32	20	4,450	25,000
11	250	193,200	328,000	672,000	28	8	3,500	33,800
13	250	234,400	344,000	552,000	18	2	2,200	17,400
15	110	136,800	280,000	408,000	10	0	750	10,100
17	130	175,200	300,000	328,000	1	0	500	6,800
19	60	176,000	284,000	624,000	1	0	550	14,000
21 ...	100	95,000	250,000	324,000	0	0	300	12,000
23 ...	70	170,000	196,000	320,000	0	....	400	6,900
25	60	152,000	248,000	288,000	....	....	140	2,200
27	50	128,000	244,000	420,000	....	....	69	1,020
29	42	152,000	164,000	282,000	....	....	30	500
31	29	114,000	150,000	228,000	....	....	5	200
33	31	120,000	132,000	210,000	....	....	3	400
35	24	150,000	244,000	154,000	....	....	0	400
37	16	136,000	240,000	224,000	....	....	2	40
39	13	34,000	124,000	70,000	....	....	1 in 2 cc.	38
41 ...	16	20,000	66,000	....	....	....	0	12
43	10	20,000	38,000	11,100	....	....	0	0

The results of this experiment not only fail to confirm the opinion that sterilizing water by heat renders it a more favorable medium for the growth of the typhoid bacillus, but shows just the opposite to be true. No explanation is offered for this, but it may be that it is due partially at least to a difference in the oxygen content of the waters. It is possible that in the process of sterilization in the autoclave enough oxygen is given off from the water to render it a less favorable medium for the typhoid bacillus than the water which was passed through the filter and not subjected to heat.

Although the window near which the bottles were set in this experiment was not as large as the one through which the light passed to the bottles in Experiment No. 1, the detrimental action of diffused daylight which was shown in that experiment was here confirmed.

Reproduction in water. — Osler's feeling of doubt regarding the possibility of reproduction in water has been shared by many writers.

Huntemüller[26] noticed an increase for a short time (about one hour) after introduction of B. typhosus into water. This he explains by assuming that a certain proportion of the organisms are in the process of fission at the time of transfer into the water, and the completion of this process already begun, he thinks, explains an initial increase in numbers.

This theory would not apply in the case of my experiments, however, as repeatedly there was increase in the number of organisms for some days. This is particularly noticeable with regard to the bacilli in the well water.

The effect on B. typhosus of the growth of other organisms in water with it.

It is generally believed that the presence of other organisms is detrimental to the typhoid bacillus in water.

For example, Thresh[27] says that "in ordinary water there exist organisms which undoubtedly exert an influence in diminishing the vitality of the typhoid bacillus." Various theories have been advanced to account for this antagonism, but none has been generally accepted.

At least one observer failed to substantiate this generally accepted belief. Daniel Konradi[28] was able to keep the typhoid bacillus as long in unsterilized as in sterilized water. He concluded that if B. typhosus be planted in water containing water bacteria, the B. typhosus will outlive the water bacteria.

Much has been written on bacterial antagonism, and in many of the experiments which have been carried on the typhoid bacillus has been used, but the greater part of this work has not been done with the use of water as a medium. The great trouble has been that no reliable method has existed for identifying the typhoid bacillus in the presence of other bacteria.

Here it might be well to review some of the methods which have been used to demonstrate bacterial antagonism.

1. Simultaneous culture on solid media. — In the use of this method alternate streaks of B. typhosus and another organism have been made on the media, either radiating from the center or crossing each other at right angles. The effect of the growth of one organism on the growth of the other was thus noticed.
2. Successive culture on solid media. — One organism has been allowed to grow on a solid media, as agar or gelatine, and then the growth has been scraped off. The other organism is then seeded on the scraped media to see if growth occurs.
3. Culture in filtered by-products. — An organism is grown in broth in flasks. Then the broth is filtered through a Pasteur porcelain filter. B. typhosus is then planted in the filtrate.
4. Cultures on opposite sides of porcelain filter. — The organisms to be tested are planted one on each side of the other in liquid media. The objection to this method is that it has been found that after a time the typhoid bacilli will go through the filter.
5. Double plate method (Frost). — A Petri dish is divided in halves by a glass rod, and sterilized. Melted agar is seeded with the presumptive antagonist and poured into one-half of the plate. Into the other half is poured sterile agar. When it has solidified the typhoid bacillus is streaked across both sides with a platinum loop. If the organism under test is antagonistic, growth of B. typhosus is inhibited on the seeded side.
6. Agar block method (Frost). — Blocks of agar are stabbed with one organism and sealed by touching with a hot iron. The agar blocks are then immersed in test-tubes of broth cultures of the other organisms.
7. Permeable sacs. — These sacs have been made of celloidin, parchment, agar films and other substances. Through these sacs dialyzable substances will pass, but not colloidal. Both broth and water have been used as media. The typhoid organisms have been seeded in the sacs in sterile or unsterile media, and the effect of other organisms in the media outside have been noted.

By these various methods antagonism to the typhoid bacillus has been established on the part of many of the common saprophytes of soil and water.

Frost[39] carried on a series of experiments in which he immersed colloidion sacs in broth. Within the sacs he seeded the typhoid bacillus, and outside, other species. These are part of his conclusions: 1, "There is a marked antagonism exerted by mixed cultures of bacteria obtained from the soil and water on B. typhosus when the same are grown in broth and a colloidion sac containing the typhoid germ is immersed therein." 2, "This antagonism results in not merely checking the growth but in actually killing the typhoid germs." 3, "There is no evidence to show that the antagonistic substances exist ready formed in the soil or water, but rather that the antagonism depends on the rapid development of the germs in the immediate presence of B. typhosus." 4, "An antagonism has been definitely associated with several different species of bacteria, viz.: B. vulgatus, B. vulgaris, Ps. fluorescens, and Ps. putida." 5, "The antagonistic substances are thermostable, being able to withstand a temperature of 120° C. for at least ten minutes." 6, "The cause of the antagonism is not due in the cases studied to the exhaustion of food supply, the action of proteolytic enzymes, specific poisons, or the production of hydroxyl ions simply."

Frost was not able to demonstrate antagonism toward the typhoid bacillus by seeding other organisms in water outside the colloidion sacs.

Russell and Fuller[30] carried on experiments in which permeable sacs were immersed in natural waters and in sewage. They used special media for the detection of the typhoid bacillus. They concluded that the longevity of the typhoid bacillus in waters is materially affected by the germ content of the surrounding water, and that when B. typhosus was exposed directly to the action of sewage bacteria, the longevity was greatly diminished.

A factor altogether unrecognized by most observers in the study of the viability of the typhoid bacillus in unsterilized waters is introduced by Otto Huntemüller.[31] He executed a series of experiments with water from different sources. These waters contained protozoa. He introduced fresh typhoid cultures, and counts invariably showed a rapid decrease in numbers of typhoid organisms, whereas there was an increase for a short time when typhoid bacilli were planted in sterile water. Huntemüller asserts that the protozoa devour the typhoid bacilli, and claims to have watched the process. He stained the typhoid bacilli with methylene blue, diluted to such a degree that they were not killed, and watched the protozoa catch, invaginate, and digest the typhoid bacilli. He concludes that the destruction of B. typhosus in water is due more to the greed of protozoa present in the water than the antagonism manifested by the water bacteria. These observations are suggestive that other low forms of life may be important factors in modifying the longevity of the typhoid bacillus in water.

In studying this problem it seemed desirable if possible to use a method by which certain definite species of bacteria could come directly in contact with B. typhosus in water. Various methods were tried with a view first to determining the presence or absence of B. typhosus in the water, and second to making comparative counts of the organism present.

1. Acid check solution. — Different quantities of hydrochloric acid and Paretti's solution were added to ten cubic centimeter amounts of sterile broth in test-tubes until an acidity was produced which allowed the growth of B. typhosus but inhibited that of common saprophytes. In this medium after twenty-four hours in the warm incubator, a cloud demonstrated the presence of the typhoid bacillus. But this method was found to be unreliable, for after living a time in water the vitality of B. typhosus was diminished to such an extent that its growth in broth was inhibited by an amount of acid which at first had no inhibiting effect.

2. Liquefaction of gelatin around colonies. — Bacillus mesentericus was plated with B. typhosus from water in the hope that by counts of the liquefying and nonliquefying colonies an estimation might be made of the number of each kind of organism present. This method was found to be impracticable, however, as the potato bacillus grew so much more vigorously than the typhoid bacillus, and its enzym acted on the gelatin so quickly that the whole plate of gelatin became liquefied before the typhoid colonies put in appearance.

3. Color of colonies. B. pyocyaneus, Blue. — It was hoped that the typhoid bacillus would appear on the plates as whitish colonies, while the pyocyaneus colonies would appear blue. But it was found that it was impossible to make use of this method, as B. pyocyaneus is such a vigorous grower, and it colors the medium diffusely without showing much color in the colonies.

S. rubrum and B. prodigiosus, Red. — With these organisms antagonism toward the typhoid bacillus appeared to be shown, but no confidence could be placed in the results, as the redness is not always distinct in the colonies of S. rubrum and B. prodigiosus, and these organisms multiplied so rapidly in the water that on pouring the agar plates the colonies of these organisms completely obscured those of B. typhosus.

4. Optimum temperature of growth. — About 37° C. is the optimum temperature for B. typhosus in agar. But at this temperature many saprophytes are markedly inhibited in growth. So it was thought that it might be possible to take advantage of this fact in making relative counts. For one reason or another it was found that the use of this method was not feasible in the case of any organism except B. carrotoverus. It was found that this organism did not form visible colonies in agar at 37° C. in two days, although it would in four days. After incubating the agar plates of this organism for two days at 37° C., the plates could be placed in the room temperature incubator and well-formed colonies would appear in two days. It was found that in plates formed from mixed seedings in water, the typhoid colonies could be counted after two days incubation in the body temperature incubators, and then after two days more in the room temperature incubator B. carrotoverus colonies could be counted. But it was found that while the typhoid organisms increased in numbers, the carrotoverus bacilli increased so much faster that it was impossible to make such a dilution that the colonies of both organisms could be counted on the same plate, and counts made from different plates would not be

accurate. So this method was used only to demonstrate the presence or absence of each variety of organism, and to give a general idea of the relative numbers.

Experiment No. 9. — Three eight-ounce bottles were nearly filled with Burlington tap water, plugged with cotton and sterilized. The water in one bottle was inoculated with one drop of broth culture of B. typhosus, a second with the same amount of B. carrotoverus culture, and a third with one drop of each. These bottles were then placed in the room temperature incubator, and every few days plates were poured. Those poured from the pure culture of B. typhosus were incubated for two days at 37° C.; those from pure culture of B. carrotoverus three days at 22° C. For typhoid growth the plates poured from the mixed culture were incubated for two days at 37° C., and those for B. carrotoverus growth for three days in 22° C. incubator. Colonies which showed growth in the warm incubator were frequently fished, and in each case after incubation in broth showed typical motile hanging drops, and responded to the Widal reaction.

The results follow: B. typhosus, pure culture. — In about three weeks the water was sterile.

B. carrotoverus, pure culture. — There was a great increase in numbers, and at the end of fifteen weeks they were still alive in increased numbers.

B. typhosus and B. carrotoverus, mixed. — Both organisms showed a marked increase in numbers, the increase on the part of B. carrotoverus being greater. At the end of fifteen weeks both organisms were alive in large numbers, the carrotoverus still exceeding.

Thus it was shown that B. carrotoverus greatly favors the reproduction and prolongation of life of B. typhosus, when seeded with it in sterile tap water.

As far as can be learned this is the only case of symbiosis reported in literature in which the typhoid bacillus is a partner.

Confirmation of these results should be attempted by repetition with similar methods and by the use of other methods. It would be interesting to learn whether or not the carrotoverus bacillus would have the same effect upon the typhoid bacillus if actual contact of the organisms were not allowed, but merely the presence of the products of growth of B. carrotoverus. This could be shown by the use of the permeable sac method.

CONCLUSIONS.

1. There is no marked difference in the behavior of the typhoid bacillus whether seeded in sterilized distilled or sterilized Burlington tap water, and neither is as favorable a medium as sterilized polluted well water.

2. There may be an appreciable reproduction of the typhoid bacillus for days after inoculation into sterile well water containing organic pollution.

3. Diffused daylight acting through a thin layer of water is decidedly detrimental to the typhoid bacilli in the water.

4. The optimum conditions in sterilized water for the development of the typhoid bacillus are a temperature of about 20°–22° C. (about room temperature), the presence of considerable organic pollution, and the exclusion of light. Under such conditions there is a marked increase in numbers, continuing rather constantly for a number of days, followed by a gradual and slow decline.

5. Not all saprophytes act antagonistically to the typhoid bacillus in water. At least one organism (B. carrotoverus, Jones) seems to manifest a symbiotic relation to the typhoid bacillus.

6. In the process of freezing there is a great reduction of typhoid bacilli in water.

7. Typhoid bacilli do not live long in ice.

8. The process of destruction of the typhoid bacilli is as marked when expulsion into the water beneath is eliminated as when it is not. Therefore, whether typhoid bacilli are or are not expelled into the water beneath in the process of freezing in natural bodies of water, it should not be considered an essential factor in the bacterial purification of water by freezing.

9. Ice harvested from ponds and lakes, even if subject to sewage contamination, probably is not a source of danger as a means of producing typhoid infection.

10. In an absolutely quiet body of water some sedimentation of B. typhosus takes place.

11. Water sterilized by filtering is fully as favorable a medium for B. typhosus as water sterilized by heat.

APPENDIX.

Analysis of Burlington tap water approximate.

Turbidity, slight.
Sediment, very slight.

Residue	Total solids	66.000
	Loss on ignition	14.000
	Fixed solids	52.000
Ammonia	Free	.006
	Albuminoid	.114
	Chlorine	1.100
	Hardness	51.000
	Nitrogen as nitrates	200.000
	Nitrogen as nitrites	000.000

Analysis of polluted well water (Alexander Well) before sterilization.

Turbidity, slight.
Sediment, considerable.

Residue	Total solids	425.000
	Loss on ignition	81.000
	Fixed solids	344.000
Ammonia	Free	.012
	Albuminoid	.154
	Chlorine	46.000
	Nitrogen as nitrates	6.000
	Nitrogen as nitrites	.028
	Bacteria	120,000.000
	B. Coli	present

Analysis of polluted well water (Alexander Well) after sterilization.

Turbidity.
Sediment.

Residue	Total solids	428.000
	Loss on ignition	100.000
	Fixed solids	328.000
Ammonia	Free	.032
	Albuminoid	.278
	Chlorine	48.000
	Nitrogen as nitrates	5.000

Sterile.

MEDIA.

The composition of the principal media used in experiments.

Bouillon: Beef extract, three grams; peptone (Witte), ten grams; water, one thousand cubic centimeters; acidity, = 1.

Plating agar: Beef extract, three grams; peptone, ten grams; agar, fifteen grams; glycerine, thirty cubic centimeters; water, one thousand cubic centimeters; acidity, 1.3 to 1.5.

Lactose agar: Beef extract, three grams; peptone, ten grams; lactose, twenty grams; agar, fifteen grams; water, one thousand cubic centimeters.

Fermentation broth: Beef extract, three grams; peptone, ten grams; lactose, twenty grams; water, one thousand cubic centimeters; reaction, .0; ten cubic centimeters of a .5 per cent neutral red solution is added.

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THE METHODS FOR TESTING THE INDOL REACTION.*

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These experiments were undertaken because of the unsatisfactory results obtained in repeatedly testing a race of B. coli communis for indol.

Some varieties of bacteria produce a varying amount of indol. By the old methods for testing the indol reaction, such a bacterium might at one time be said to produce indol and not at another. This variation is mentioned by Gordon.[1]

The methods of applying the indol test as ordinarily used in laboratories and described in text-books are many and various.

The media commonly employed are:

- Sugar-free bouillon with one per cent peptone and one-half per cent NaCl — Smith.[2]
- Sugar-free bouillon with one per cent peptone and no NaCl.
- Peptone solution (Witte) with one per cent peptone and one-half per cent NaCl — Dunham.
- Peptone solution (Witte) with one per cent peptone and no NaCl.
- Alkali-peptone bouillon — Peckham.[3]
- Sugar-free bouillon without peptone — Smith.

The number of days' growth in such media is stated as follows:

- Three days' growth — Peckham.
- Two days' growth.

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* Received for publication May 2, 1906.

Twenty-four hours' growth — Grubbs and Francis.[4]
Sixteen hours' growth — Smith.

The amounts of H_2SO_4 used are as follows:

A varying amount of a twenty-five per cent H_2SO_4 — Peckham.
One drop concentrated H_2SO_4 — Muir and Ritchie.[5]
Ten drops concentrated H_2SO_4 — Grubbs and Francis — Smith.

The strengths of $NaNO_2$ are:

1–1000 — Grubbs and Francis.
.01 per cent — Smith.
.02 per cent — Chester.[6]

The methods of applying the acids and nitrites are:

Add the nitrite and then the acid — Muir and Ritchie.
Add the acid, shake, and allow the nitrite solution to run down the tube carefully so as to form a line of contact — Grubbs and Francis.

MEDIA. — In a large number of experiments carried on to determine the best method, it was soon found that the bacteria grew better when sodic chloride was present. In testing the sugar-free bouillon by the old method of Muir and Ritchie, the color was deeper in this medium than in Dunham's peptone solution in three series of tubes only, while in the greater number of tubes the color was less marked. B. mucosus capsulatus grows in sugar-free bouillon with a heavy pellicle, and the edge of this interferes with seeing the reaction when the ring test is used. The color fades quicker in sugar-free bouillon. The alkali-peptone bouillon of Peckham was not used because Dunham's peptone solution was very satisfactory and easier to make.

Experiments with different peptones have already been carried on by Voges and Proskauer[7] and Wherry.[8]

Sugar-free bouillons without peptone gave no indol reaction.

TIME OF GROWTH. — The color is deepest in a three-days' growth. All bacteria examined gave an indol reaction by the ring method in twenty-four hours except typhoid, and two paratyphoids. The other bacteria examined were B. aerogenes, B. coli communis, B. neapolitanus, B. mucosus capsulatus, B. pneumoniæ, B. cloacæ, B. acidi lactici. Typhoid gave a reaction in two days, and the two paratyphoids gave a faint ring in three days. The rings in the twenty-four-hour cultures of B. mucosus capsulatus, B. pneumoniæ and B. cloacæ were faint also. No experiments were carried on in any growth less than twenty-four hours old as it did not seem practical.

AMOUNT OF ACID. — In testing the effects of acid, Peckham's indefinite method could not be used. One drop of concentrated sulphuric acid in a twenty-four-hour growth of B. coli communis gave a faint ring, but it was slow in forming. Ten drops of the acid gave a better reaction, twenty drops a deeper color, thirty drops a still deeper color, and one drachm a well-marked reaction. The tests with ten drops and stronger were also carried on with bacteria giving [illegible] ith the same results. [illegible] e in the Dunham's peptone solution [illegible] Ten drops gave almost no color ring, [illegible] own ring, twenty drops a brown ring and just below it a faint pink ring, thirty drops a brown ring and a deeper pink ring below it, sixty drops a well-marked brown ring and a pink ring below. This shows that fifteen drops and more of the concentrated acid interfere with the reaction.

STRENGTH OF SODIC NITRITE. — Sodic nitrite was first used in the strength of 1–1000. This strength gave an upper yellow ring and a lower pink ring. The yellow color interferes with faint reactions. A .01 per cent solution was next used, but the reaction was faint and the ring slow in forming. The .02 per cent solution gave a reaction almost immediately after adding it, and gave no well-marked yellow ring.

It is interesting to note in this connection that there was no yellow ring in the sugar-free bouillon without peptone, when the 1–1000 $NaNO_2$ solution was used.

The use of the method in the Muir and Ritchie text-book gives the confusing results mentioned in the beginning. The ring method as suggested by Grubbs and Francis, with certain modifications, is the best and should be uniformly used.

In all the above experiments transfers were made from twenty-four-hour growths on agar, blood serum, to the indol media.

CONCLUSIONS.

1. Dunham's peptone solution with salt is best and quickest made.
2. A twenty-four-hour growth should be used; if this is negative, a forty-eight or seventy-two hour growth.
3. Ten drops of concentrated H_2SO_4 should be added with a dropper and the tube shaken.
4. Thirty drops of a .02 per cent sodic nitrite should next be carefully added, drop by drop, with the tube held almost horizontal.
5. The intensity of the color ring should be noted, whether faint or well marked.

The above differs from the rules laid down by Grubbs and Francis, in that it does not set the limit of growth at twenty-four hours, uses a weaker sodic nitrite solution and makes a distinction in color between well marked and faint.

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THE CHOLERA–RED REACTION AND THE INDOL REACTION.

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Wherry speaks of the distinct difference between the violet or purple-colored indol reaction, and the vermilion-colored cholera-red reaction. In Dunham's peptone solution these colors are better stated; the mauve indol reaction and the violet-red cholera-red reaction.

Having determined the most satisfactory method for testing the indol reaction, it seemed fitting to try the method on the spirilla. To twenty-four-hour growths of Sp. choleræ, Sp. Metchnikoff, Sp. Finkler-Prior and Sp. Deneke, ten drops of concentrated H_2SO_4 were added and the tubes shaken. The diffuse violet-red color appeared in the Cholera and Metchnikoff tubes only. The color was deeper in the cholera tube. Thirty drops of a .02 per cent sodic nitrite solution was added carefully drop by drop with the tube held almost horizontal. The usual red indol ring [illegible] in all the tubes but the Deneke. Thus, in the [illegible] Metchnikoff tubes there is the diffuse red-violet [illegible] sharp red ring of indol.

[illegible] growth the acid brings the diffuse red-violet color in the Cholera and Metchnikoff tubes, and a faint tint of the same color in the Finkler-Prior tube. On adding the sodic nitrite the red indol ring appears in all tubes with the diffuse color as above stated.

In a three-day growth the acid brings the diffuse red-violet color in the Cholera and Metchnikoff tubes, and a faint tint of the same color in both the Finkler-Prior and Deneke tubes. On adding the sodic nitrite the red indol ring appears in all tubes with the diffuse color as above stated.

All the tubes gradually lose the diffuse color on standing, the cholera being the last to lose it, and the red indol ring deepens in color and becomes wider.

The Metchnikoff spirillum loses its color most quickly;

the violet gives way to a bright yellow, and the indol ring deepens and becomes wider.

Experiments with the sugar-free bouillon of Smith show that this medium gives a fainter reaction and the color disappears quicker.

Another method of testing the difference between the cholera-red reaction and the indol is to underlay the peptone solution with concentrated H_2SO_4, care being taken in putting the acid at the bottom with a pipette that no acid is on the outside of the pipette. In a two-day growth of B. coli and Sp. choleræ the results were as follows: In the colon tube, after adding the nitrite solution, there was a dark brown ring at the line of contact with a pink ring above it; in the cholera tube the brown ring was absent, and there was a thin layer of violet-red cholera-red above the line of contact.

CONCLUSIONS.

The cholera-red reaction is different from the indol reaction for the following reasons:

1. The color is different; cholera-red is a red-violet, indol is a mauve.
2. After the cholera-red is brought out by acid, the indol reaction may be produced by adding the nitrite.
3. When the nitrite is added to the colon tube, but not to the cholera tube, and acid introduced at the bottom, the rings produced are different.*

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* These two tests serve to distinguish the different varieties of spirilla from one another.

DESCRIPTION OF PLATE XXIV.

(*Original by Mrs. C. G. Page.*)

All tubes are twenty-four-hour growths in Dunham's peptone solution.

FIG. 1. Cholera spirillum. There is the diffuse red-violet of the cholera-red and the red ring of the indol.

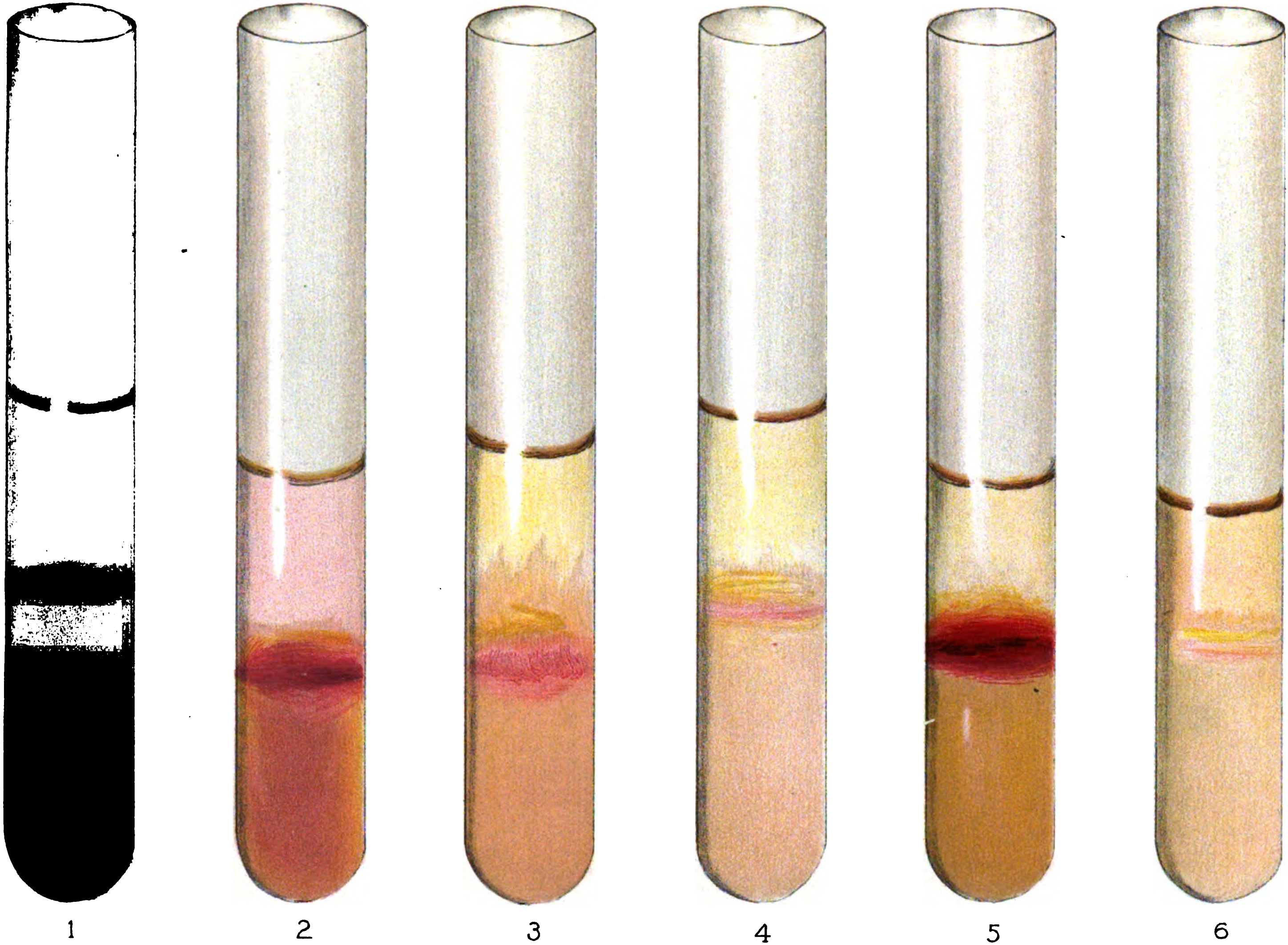
FIG. 2. Metchnikoff spirillum. There is a fainter diffuse red-violet and a deeper red ring of indol.

FIG. 3. Finkler-Prior spirillum. There is a very faint diffuse red-violet and a well-marked red ring of indol.

FIG. 4. Deneke spirillum. There is practically no diffuse violet, but a faint red ring of indol is present.

FIG. 5. Metchnikoff tube at the end of two hours. The red-violet has given way to a yellow, the indol ring has become a deeper red and is wider.

FIG. 6. A tube of the Dunham solution with no growth. At the end of an hour there is a barely perceptible red ring when tested by the ring method for indol.



Tobey.

Indol reaction

THE URICOLYTIC ENZYME IN ANIMAL ORGANS.*

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DECOMPOSITION OF URIC ACID AND XANTHIN BODIES BY ANIMAL ENZYMES.

(*Preliminary Considerations.*)

As our knowledge of enzymes and their action increases, we become more and more assured that the greater part, if not all, of the chemical processes of the body take place through their agency. Formerly we were content to regard them as instrumental only in the process of digestion and to consider that they were secreted only in the digestive tract; now we know that they are found in all the cells, and those chemical changes, which were regarded as vital processes of the cell, we now consider due to enzymatic action. Glycolysis, autolysis, oxidation, fermentation, catalysis are all actions of appropriate enzymes secreted by cells and undoubtedly for various other vital processes we shall soon find special agents of a similar character. From the crude process of mixing organic substances with macerated organs, the advance to isolation and characterization of the specific enzyme has been a rapid one until now, though no universal method of isolation applicable to all has been found, yet some scheme particular to each has been discovered by which we may extract the active agent from organs and employ it in its most concentrated form.

Since 1848 it has been known that warm blooded animals have the power of destroying uric acid as only a small portion of that ingested reappears as acid, the remainder as urea. More recently Weintraud[1] has confirmed beyond all doubt that this is true; further, that the power varies in animals, being greater in dogs than in rabbits, while in the puppy the power is practically absent, increasing with the age of the animal. Another fact, which has still further demonstrated this action, lies in the inability to find an

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amount of uric acid in the urine which would correspond to the amount of basic nitrogen in nuclein which is taken and which forms the mother substance of this acid; when we accept the theory, however, that the body cannot only build but destroy uric acid then this discrepancy is explained. Still further proof of this action was advanced by Burian and Schur,[2] who found that when they injected a definite amount of uric acid in an animal they produced the same increase of that substance as when they injected or gave by mouth an equivalent amount of purin bases. The most plausible explanation of this phenomenon is, in their mind, that the purin bases are converted into uric acid of which a portion is destroyed, while the balance is eliminated just as in case of injected or ingested uric acid. From this must be concluded that uric acid is not the end product but an intermediate product of the destruction of the nucleins. The next question which presents itself is, where does this destruction of the acid take place? Stokvis,[3] Jakoby,[4] and Wiener[5] have all shown that uric acid may be destroyed by mixing it with macerated dog's liver or an extract of the same, while Ascoli[6] has shown the same result by passing blood charged with this acid through the liver in situ. Pig's liver has demonstrated the possession of the same power. The kidneys likewise possess a similar power. Here the question rested until Jones[7] began his most interesting series of researches in which he demonstrated that by autolysis of the thymus gland and adrenals their natural adenin and guanin are converted into xanthin and hypoxanthin in traces, while the adenin of the spleen is converted into guanin and hypoxanthin.

Jones found that if guanin be added to the products of self-digestion of the pancreas the xanthin was already increased and the guanin disappeared. This he attributed to an enzyme which he called guanase. Later he also found an enzyme in the spleen which would convert adenin into hypoxanthin, which was called adenase.

Schittenhelm[8] not only confirmed the researches of Jones, with the exception of identifying distinct enzymes for the conversion of adenin and guanin, but found that the process was carried still further to the production of uric acid. He found that both adenin and guanin when digested with spleen extract were converted almost quantitatively to uric acid. Extracts of liver and spleen had the same effect. Furthermore, by the aid of ammonium sulphate, this enzyme could be separated and was very efficient. This author was, however, unable to obtain at all times an amount of uric acid corresponding to the amount of guanin which was added to the extract, so that he was led to think that a portion of the uric acid was destroyed by an enzyme — which proved to be true. When half a gram of uric acid was added to four hundred cubic centimeters of extract and digested at 42° C., with a current of air passed through it for four days, no acid could be found in the mixture.

In a later communication of Schittenhelm, devoted entirely to the so-called uricolytic ferment, the author states that he has found this enzyme in the kidney, liver, muscles, and possibly in the bone marrow, but not in the spleen, lungs, and intestines — which can form but not destroy uric acid. He was also able to isolate the ferment from bovine kidney and with it forty-six to one hundred per cent of the added uric acid was destroyed when the unheated solution of the enzyme was used ; when the solution was heated to 80–100° C., no change took place in the acid.

Burian [9] also has found that extracts of ox liver have the power to form uric acid from the purin bases as well as to decompose uric acid, but these enzymes are distinct from each other.

In my own experiments the method of Jakoby [10] was used, which is as follows:

The organs were finely minced in a meat cutter and then extracted with three times their weight of toluol water, for forty-eight hours, stirring occasionally; the mixture was first passed through gauze and then filtered with a suction filter; a clear solution resulted, which was precipitated with two parts of saturated ammonium sulphate solution to one of the extract. The precipitate was filtered out, dissolved in a liter of water and dialyzed against running water until no more ammonia could be detected. It was then placed on ice and used as required for the experiments.

After the digestion was completed all solutions were boiled and, while boiling, diluted acetic acid was added until acid, when the coagulum settled out, leaving a clear albumin-free solution. The uric acid was then isolated by the method of Salkowski, only that the final precipitate of uric acid was destroyed and the resulting nitrogen found multiplied by three and the usual correction for solubility in filtrate and wash water added.

Experimental.

Experiment 1. — Two ox spleens weighing 2,200 grams plus 8 liters toluol water extracted seventy-two hours, passed through cloth and filtered.

(*a*) 500 cc. of the watery extract alone placed in brood oven at 38° C. for seventy-two hours, protected from putrefaction by toluol and a drop or two of chloroform.

(*b*) 500 cc. watery extracts plus .4 gram urate of soda, brood oven same time.

(*c*) 500 cc. extract plus .45 gram uric acid dissolved in 20 cc. sodium hydroxid, for same time in brood oven.

Result. — No crystals of uric acid were found in either of the solutions, and no nitrogen was found in the solution from which the attempt was made to isolate the uric acid by hydrochloric acid and concentration.

Experiment 2. — 3,000 cc. of the above watery extract precipitated with 6,000 cc. saturated ammonium sulphate and filtered. The precipitate was treated as described above and the solution containing the isolated enzyme employed. As suspicions were aroused in my mind that the alkali used in dissolving the uric acid may have been responsible for its destruction at brood oven temperature four solutions were prepared as follows:

(*a*) 400 cc. of the isolated enzyme extract with toluol and chloroform.

(*b*) 400 cc. of extract plus .5 gram uric acid in 100 cc. normal sodium hydroxid with antiseptics.

(*c*) 385 cc. extract plus .5 gram uric acid in 100 cc. with the usual antiseptics.

(*d*) 400 cc. water with .25 gram uric acid dissolved in 100 cc. normal sodium hydroxid.

All were kept in the brood oven for ninety-six hours.

Result. — Solution (*a*) contained no uric acid and no nitrogen; (*b*) contained no acid; (*c*) contained .0084 gram uric acid; while (*d*) the control contained no acid.

From the result in the uric acid solution alone it will be at once seen that a very serious error in the interpretation of the results of the experiments may occur — as 100 cc. of normal sodium hydroxid in 400 cc. of water at a temperature of 38° C. can destroy .25 gram of uric acid. Hence in all the subsequent experiments a control of the acid alone in the same amount of sodium hydroxid which was used for the others was always employed. It did not seem advisable to use the acid undissolved since it is an accepted axiom that an enzyme does more work when acting on a soluble substance: an example of this will be found in the action of pepsin on acid albumin and of the stomach steapsin on emulsified fat.

Experiment 3. — 2,250 grams finely divided ox spleen were extracted two days with five liters toluol water with occasional stirring. The enzyme, isolated as usual, was dialyzed five days. Resulting solution 1,200 cc. The four solutions consisting of

(*a*) control 400 cc. extract without uric acid.

(*b*) 400 cc. extract with .5 gram acid in 20 cc. N/2 sodium hydroxid.

(*c*) ditto.

(*d*) Same amount of water with an equal amount of uric acid and alkali. All the mixtures were kept in the brood oven for six days, with the usual protection against putrefaction.

Result. — (*a*) contained no uric acid; (*b*) contained .2429 gram, here .2571 gram was destroyed; (*c*) contained .16629, hence .3338 gram was destroyed; (*d*) contained .1925 gram, hence .3075 gram was destroyed. When we look at these results more closely we see that the sodium hydrate alone was as efficacious in destroying the acid as the alkali plus the splenic extract.

Experiment 4. — 25,40 grams beef kidneys were extracted with 4,000 cc. toluol water. Sedimented and decanted the supernatant fluid was used for these mixtures without filtration. Four mixtures were prepared as above with the same amounts of each ingredient. Digestion time forty-eight hours.

Result. — (*a*) contained .0119 gram uric acid; (*b*) .1007 gram; (*c*) .1048 gram; and (*d*) .1914 gram.

Two things are noticeable here: first, that the kidney contains quite an appreciable amount of uric acid which was always absent in the spleen, and second, there is clear evidence of an enzymatic action in this renal extract, for, taking the average of (*c*) .10279 and (*d*) .1914, we have .0887 gram more destroyed by the action of the extract and the alkali than by the alkali alone, to which must be added the .0119 gram which existed in the original renal extract, a total of .1006 gram.

PRODUCTS OF DECOMPOSITION OF URIC ACID BY ANIMAL ENZYMES.

(*Preliminary Considerations.*)

We come now to the products formed by the splitting of uric acid in the laboratory, which are as follows:

(1.) If we heat uric acid with concentrated hydrochloric acid in a closed tube at 170° we obtain with addition of water, *i.e.*, hydrolysis, glycocoll, CO_2 and NH_3.

(2.) If we use nitric acid for the purpose of splitting we obtain in the cold alloxan, and, by warming, this is converted into parabanic acid and carbon dioxid. The parabanic acid takes on water and is changed into oxaluric acid, while the latter after taking on a second molecule of water is split into oxalic acid and urea.

(3.) By the action of potassium permanganate in the cold, uric acid is split into allantoïn and carbon dioxid and by the further action of an oxidizing substance on allantoïn we obtain urea and oxalic acid, which latter eventually becomes split to carbon dioxid. Of all these substances — urea, oxalic acid, allantoïn and glycocoll are found in the body, and hence it is reasonable to suppose that any or all may be found as the result of the action of animal enzymes on uric acid. After going over the literature carefully, Wiener[11] comes to the conclusion that glycocoll is the only substance that has been proved to be a decomposition product of uric acid in the body.

The author,[12] however, in an investigation of the sources of oxalic acid found that when urine is allowed to decompose the oxalic acid increases at the expense of the uric acid, while Swain[13] found that a small portion of ingested uric acid in dogs appears in the urine as allantoïn. His explanation was that the conversion was carried beyond the stage of allantoïn. In all the following experiments careful efforts were made to extract both oxalic acid and allantoïn from the digested mixtures after the uric acid was removed.

METHOD OF ISOLATION. — The allantoïn, as is well known, is precipitated by silver as well as the uric acid, but the former silver compound is soluble in an excess of ammonia which does not affect the uric acid silver compound. Hence the filtrate from the uric acid precipitation was acidified and freed from silver by means of sulphureted hydrogen, and since allantoïn is insoluble in ether and alcohol, the filtrate was repeatedly shaken in a separating funnel with that substance, after being strongly acidulated by hydrochloric acid, the ethereal extracts united, the ether driven off and the residue treated as recommended by Salkowski[14] for oxalic acid in urine. The fluid after extraction with ether-alcohol was freed from these by warming and treated with mercuric nitrate, the precipitate, if any were formed, was suspended in water, the mercury removed by sulphureted hydrogen, the filtrate concentrated and allowed to stand in a cool place. It was then examined microscopically.

For comparison, uric acid was decomposed in the cold by permanganate of potassium, one molecule of the former to three of the latter, the uric acid left removed by silver, freed from silver, concentrated and precipitated by acetic acid. The characteristic long prismatic crystals arranged in stellate form were readily found.

Experiment 5. — 1,500 cc. of the toluol water extract of macerated ox kidney precipitated with ammonium sulphate, dialyzed five days, resulting solution 1,200 cc. Four mixtures as before: (*a*) 400 cc. extract alone; (*b*) and (*c*) 400 cc. extract plus .5 gram uric acid in 20 cc. normal sodium hydroxid; (*d*) 400 cc. water plus .5 gram uric acid in 20 cc. normal sodium hydroxid. Time of digestion three days.

Result. — (*a*) contained no uric nor oxalic acid and no allantoïn; (*b*) contained .175 gram uric acid; and (*c*) .0889 gram of the same, but neither had oxalic acid nor allantoïn; (*d*) contained .18779 gram uric acid and both oxalic and allantoïn. In (*b*) we find results but little better than in (*d*) where the destruction of the acid was brought about by the alkali alone. In (*c*), however, .0992 gram more was destroyed by the combined action of both extract and alkali than by the latter alone. There can be only one explanation for the non-appearance of allantoïn and oxalic acid in the extracts, and that is that the process was carried much farther than these products.

Experiment 6. — 2,530 grams macerated ox spleens, extracted with 5 liters toluol water, precipitated as much as possible with dilute acetic acid and decanted. Supernatant fluid used for digestion. Four solutions prepared as before: (*a*), (*b*), (*c*), and (*d*). Time of digestion six days for (*a*), (*b*), and (*d*), while (*c*) had air passed through it for twenty-five hours at a temperature of 38° to 40° C.

Result. — (*a*) contained nothing; (*b*) contained .00899 gram uric acid, some typical allantoïn crystals, but no oxalic acid; (*c*) contained .00389 uric acid, no oxalic acid, and no allantoïn, but abundant tyrosin, probably due to the difficulty of keeping the mixture aseptic on account of the volatility of toluol and chloroform in a current of air. Tyrosin, as is well known, is an early product of putrefaction of albuminous material; (*d*) contained .07079 uric acid and no oxalic acid but typical crystals of allantoïn. The destruction of uric acid, as will be seen, is not increased by the passage of air but very decidedly so by the longer duration of the digestion; the effect of the alkali and the extract is most emphatically greater than the alkali alone.

Experiment 7. — 2,000 cc. of the toluol water extract was precipitated with ammonium sulphate, dialyzed for five days. Four digestions prepared as before. Time of digestion in brood oven for (*a*), (*b*), and (*d*), six days. Air was passed through (*c*) for thirty hours at a temperature of 38° to 40° C.

Result. — (*a*) contained nothing; (*b*) contained .0894 gram uric acid and some oxalic acid; (*c*) contained .143 gram uric acid, oxalic acid, and allantoïn; (*d*) contained .0769 uric acid, oxalic acid and allantoïn. The results with the alkali alone are equally as good as those of both alkali and extract in one, and much better than in the other.

CONCLUSIONS.

In all these experiments we have some evidence that there is a uric acid splitting enzyme in both the spleen and kidney of the ox; still the results obtained are so closely simulated by the alkali used for solution of the uric acid that the proof is not positive. True, we must consider that the alkali mixed with a solution which is strongly albuminous by its combination with protein to form alkali albumin is rendered much less efficacious in its action on the acid than the sodium hydroxid alone; that there is such a formation of alkali albumin is shown by the fact that in the mixture, when digestion is completed, a copious precipitate can be thrown down by dilute acetic acid, which is soluble in excess. Schittenhelm, cited above, says that this destruction of uric acid cannot be due to the action of alkali alone since he was able to isolate the same amount which he introduced when the mixture had been boiled for a moment before digestion. Hence it is possible that the albumin inhibits the action of the alkali upon the acid: yet Nencki and Sieber[15] have shown that uric acid is converted to uroxantic acid by very dilute alkali solutions at blood temperature. How, then, are we to avoid the effect of the alkali used for solution which is apparently set free as the uric acid is decomposed?

We must either find some other solvent for the acid or, as stated before, use it in substance, and by constant agitation attempt to keep it in suspension.

May we not, with equal justice, ask how much of the action of the guanase and adenase is due to the alkali used as a solvent by Schittenhelm? If this action is due to an enzyme it is extremely variable, but much more active apparently when the simple watery extract is used than when attempts are made to isolate it by precipitation. Furthermore, time had little effect, equally good results having been obtained at times in three as six days' digestion with the watery extract, while at others the longer period produced the better results.

The use of air did not appear to increase the action of the extract, so that it cannot be purely an oxidation process.

The results show that splenic extract is fully as active as renal extract in its uricolytic function, though Schittenhelm did not find the action in the spleen. It is fully established that uric acid is broken up into allantoïn and oxalic acid both by weak sodium hydroxid (2:1000) at a temperature of 38° to 40° C., and also probably by splenic extract; that is, by an enzyme.

My attention is now directed to the isolation of the enzyme from human organs, where I hope to overcome some of the present difficulties.

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NON-VIRULENT DIPHTHERIA BACILLI FROM INFECTED MIDDLE EAR.*

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A smear from a culture taken from an infected middle ear showed the following: When stained by Lœffler's methylene blue, slender rods were present having darkly stained granules in them, and there were also club-shaped forms; when stained by Neisser's method, the rods were of a faint brown color with blue granules in them. The rods had the characteristic morphology and arrangement of diphtheria bacilli. Four competent observers saw the bacteria and said they were diphtheria bacilli. A transfer from this culture was made to bouillon and two cubic centimeters of this nearly pure culture injected into a guinea-pig. Pure cultures were made, and three days later, as the pig was not ill, two cubic centimeters of a pure bouillon-culture of forty-eight hours' growth were injected. Two days after the last inoculation the pig seemed the same as usual except for a slight swelling at the site of inoculation. The pig was then killed and at autopsy an abscess was found at the site of inoculation. Smears from the abscess showed diphtheria bacilli. The suprarenal capsules were light-colored and normal, and there was no swelling of the lymphatic glands in the groin or peritoneum. An injection of four cubic centimeters of a pure culture of forty-eight hours' growth was made into another guinea-pig, but the pig showed no sign of illness.

Transfers were made from a pure culture of the bacillus to serum-water-litmus-dextrose, lactose, and mannite tubes. Two days later the dextrose was acid, but the mannite and lactose remained unchanged even after ten days' incubation, and the dextrose remained acid.

The cultures were destroyed in cleaning the room and further work prevented.

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SUMMARY.

1. The bacilli had the characteristic morphology, staining reactions, and arrangement of diphtheria bacilli.

2. The bacteria were non-virulent to two guinea-pigs, even when four cubic centimeters of a pure culture of forty-eight hours' growth were injected.

3. The bacilli turned dextrose-serum acid, but did not affect lactose or mannite. Virulent B. diphtheriæ ferment mannite with acid production (Knapp).

CONCLUSION.

This bacillus differs from the type B. diphtheriæ not only in virulence but in its action on sugars.

THE INFLUENCE OF SUBCUTANEOUS INJECTION OF LIVER EXTRACT UPON THE HEPATIC CATABOLISM OF URIC ACID.*

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In an earlier paper the writer[1] in collaboration with A. S. Granger pointed out that subcutaneous injection of liver extract (dog) may lead in the case of the dog to an increased elaboration of nitrogenous end-products into urea. From the fact that liver extracts which had been heated to 55° C. were not found to possess this stimulative action, attention was directed to the possibility of an enzymatic formation of urea.

In view of these results regarding the formation of urea, it has seemed of interest to study the influence of this same treatment — namely the subcutaneous injection of liver extract — upon the hepatic metabolism of uric acid.

It long has been known that the animal organism is able to destroy uric acid. Frerichs and Woehler[2] fed uric acid to dogs and rabbits and found in the urine only a small part of the uric acid ingested. Stokvis[3] was the first to note that if uric acid is added to a freshly hashed liver a considerable portion of the uric acid disappears. Ascoli[4] showed that if defibrinated blood containing uric acid is perfused through a surviving liver there is a progressive loss of uric acid. The capacity of the various isolated organs to destroy uric acid has been investigated directly by Brunton and Bokenham,[5] Wiener,[6] Jacoby,[7] Schittenhelm,[8] Burian and Schur,[9] Croftan,[10] Almagia,[11] and others. From the researches of these investigators it seems that the liver, kidney, marrow, muscle, spleen, lymph glands, intestinal wall, and blood possess a varying ability to catabolize uric acid. In herbivorous animals the most active organ weight for weight in destroying uric acid is the kidney; and in carnivora and omnivora the liver. Burian and Schur, and

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Croftan have called attention to the possibility that although the uric acid destroying action of the muscular tissue is weak, on account of the great bulk of body muscle, it may play an important part in the catabolism of uric acid.

In omnivora the importance of the liver in the catabolism of uric acid is illustrated well in the observations of Hahn, Massen, Nencki and Pawlow[12] after the establishment of the Eck fistula. These observers found that when the liver is thrown out of circulation a larger quantity than usual of uric acid escapes oxidation and is eliminated directly in the urine. The increased excretion of uric acid in cirrhosis of the liver may offer further evidence of the importance of this organ in the destruction of uric acid.

Wiener,[13] Schittenhelm,[14] and Croftan[10] have shown that the ability of the kidney and liver to destroy uric acid is dependent upon the activity of an enzyme which has been termed appropriately a "uricolytic ferment." Wiener[13] noticed that a fresh extract free from cells possesses less uricolytic power than the cells themselves. Almagia[11] has shown that it is not possible to filter the ferment from the fresh organ by means of a Buchner press.

Recognition of the ability of the organism to catabolize uric acid like other nitrogenous substances has changed profoundly our conception of purin metabolism, and since in carnivora and omnivora the liver probably is the most important organ in the destruction of uric acid, the need of further study of hepatic metabolism of uric acid is apparent.

The extracts used in these experiments were prepared from livers taken with the usual aseptic precautions from dogs by hashing immediately as fine as possible and shaking with five parts of physiological salt solution. Toluol was added to prevent bacterial action. These extracts were kept at room temperature, and the supernatant fluid used without filtering. At the beginning ten cubic centimeters were injected subcutaneously daily, and the amount increased gradually to fifty cubic centimeters. Throughout the experiments the dogs were allowed food and water freely. At the conclusion of each experiment the uricolytic power of the liver was determined by hashing the organ finely, adding weighed portions of uric acid solution of known strength, keeping at 37° C. for a definite length of time in the presence of toluol, and then by estimating the amount of uric acid which remained. In each case the liver of a normal dog was examined under identical conditions. Uric acid was determined by the method of Folin and Schaffer.[15]

The results obtained are given in the following table:

TABLE OF RESULTS.

No. of Exper.	Nature of Liver.	Liver taken.	Vol. uric acid solution.	Strength uric acid solution.	Time of exposure.	Uric acid. destroyed.		Uric acid destroyed, terms 100 grams liver.
		grms.	*cc.*	%	*hrs.*	*grm.*	%	*grm.*
1.....	From normal dog.	40	1,000	0.0255	42	0.085	33	0.212
	From dog treated 5 days.	39	1,000	0.0255	41	0.105	41	0.269
2.....	From normal dog.	25	500	0.05	72	0.056	22	0.224
	From dog treated 6 days.	25	500	0.05	71	0.066	28	0.264
3.....	From normal dog.	15	500	0.063	96	0.092	28	0.613
	From dog treated 7 days.	15	500	0.063	95	0.098	31	0.653
4.....	From normal dog.	50	500	0.071	24	0.077	21	0.154
	From dog treated 8 days.	50	500	0.071	23	0.081	23	0.162

These results show a noticeable increase in the uricolytic power of the liver of treated dogs over that of normal animals.

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MODIFICATION OF BACILLUS DYSENTERIÆ AFTER CULTIVATION IN AGGLUTINATING SERUM.*

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As is well known, Ehrlich's theory attempts to explain the phenomena of the infectious diseases by assuming that these processes have an ultimate chemical as well as biological basis. The circulating toxin unites chemically through its haptophore group with the corresponding haptophore group of the body cell. When it is incorporated in the cell, the toxin may produce its specific lesion by means of its active toxophore chemical group. If the infected animal recovers, it protects itself against toxins by forming antitoxins. The antitoxins are nothing else than susceptible haptophore groups of the body cells which have been formed in excess as the reaction of the cell to the toxin, and have been finally set free in the circulation.

In the Huxley lecture in 1902, Dr. William H. Welch[15] suggested that the reactions occurring in infected animals, which lead to their immunization, are not unique, but have their counterparts in the reactions occurring in the invading microörganisms themselves. In other words, he proposed to apply the principles of Ehrlich's receptor theory to explain the reactions occurring in the infecting bacteria, as well as those of the infected animal. In this address, Dr. Welch used the following language: "I see no reason why suitable substances derived from the host may not stimulate parasitic organisms, through a physiological mechanism similar to that operative in the development of cytolytic immunity, to the production of intermediary bodies, which, if provided with the requisite affinities, have the power to link complements to cellular constituents of the host and thereby to poison the latter. Expressed in terms of Ehrlich's side-chain theory, certain substances of the host of cellular origin,

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assimilable by the parasites through the possession of haptophore groups with the proper affinities, become anchored to receptors of the parasitic cell, which, if not too much damaged, is thereby stimulated to the overproduction of like receptors; those excessive receptors of the parasite, if cast off into the fluids or the cells of the host, there constitute intermediary bodies or amboceptors with special affinities for those cellular constituents or derivatives of the host which led to their production and for others which possess in whole or in part identical receptors. Provided the host is supplied also with the appropriate complements, there result cytotoxins with special affinities for certain definite cells or substances of cellular origin in the host. The contribution of the parasitic cells to those cytotoxins is the amboceptors. Either the parasite or the host may provide the complements.

"It may perhaps aid in grasping the ideas here presented to imagine the bacterium, in the capacity of the host, as a structure so large that one could inject into it animal cells. Provided the proper receptor apparatus is present, the resulting reaction on the part of the bacterium, as described, would be a process of immunization against the animal cells through the formation of specific cellulicidal substances. In reality it is only certain atomic complexes of cells which are concerned in this immunizing reaction, and in comparison with these even the smallest bacterium is a gigantic object."

Experiments started at the time that this lecture was delivered, by one of the writers, gave some support to Dr. Welch's hypothesis. For example, typhoid bacilli grown for several transfers in bouillon, to which was added rabbit's blood, appeared to exert a greater action upon the red-blood corpuscles in the later transfers. These experiments, unfortunately, had to be stopped before definite conclusions could be drawn from them.

During the summer of 1903 an opportunity offered to resume this line of research, and the problem was approached in a different way.

Reasoning from the hypothesis advanced by Dr. Welch, it is seen that when an animal is immunized by inoculation with a toxin, the material injected is naturally incapable of reacting in any way, while the inoculated animal undergoes a series of changes, which finally render the animal immune. On the other hand, if the animal is inoculated, not with a toxin, but with a living culture of bacteria, not only does the animal react, but it is conceived that the bacteria also react to the stimulus of their environment.

In an analogous way it may be supposed that bacteria may be immunized if they are grown in the presence of sub-lethal amounts of some injurious serum. Here, the serum would be incapable of reacting, while the living bacteria would be in a position analogous to that of an inoculated animal. The condition would be similar if, instead of taking a normally injurious serum, the bacteria were placed in the serum from an animal previously immunized against the bacterial species. This serum would contain: 1st. Any normal agglutinins, and the normal bacteriotoxins; 2d. The specific agglutinins and bacteriotoxins called forth in the immunized animal by the inoculated material; 3d. A long series of other antibodies of the first, second, and third orders proposed by Ehrlich, and finally a whole array of other substances normal to the serum, with which we need not concern ourselves.

For our experiments we selected Bacillus dysenteriæ of the "Flexner-Harris" or "acid" type, as the bacterial species to "immunize," and we grew the bacteria in the presence of serum from horses immunized against this species. The extent to which the bacteria were "immunized" was measured by the degree of their reaction with the agglutinins in the specific serum from the horse. Our attempt, then, was to immunize the bacteria in such a way as to protect them from the agglutinins found in the serum of immunized horses.

The technic of the first series of experiments was as follows:

A large amount of bouillon was made, sufficient to last for a whole series of experiments. To a series of test-tubes, containing each one cubic centimeter of bouillon, was added the agglutinating serum in quantities varying from two cubic centimeters to .01 cubic centimeter. The volume of fluid in the test-tubes was in each case made uniform by the addition of sterile normal salt solution. The stock culture of the Flexner-Harris bacillus was grown for eighteen hours in bouillon, and from this a few drops (one to six) were dropped into each test-tube, the amount for each experiment being constant. As a routine method three drops were employed. In some experiments one drop was used, in others six drops. In the later experiments a few platinum loopsful only were inoculated. After the tubes were inoculated, controls were made of the bouillon suspension used for inoculation. Litmus milk, litmus glucose agar, litmus mannite agar, bouillon, agar plate cover-slips and hanging drop preparations were used for the controls. This series was placed in a thermostat for a day and the results noted. A new series, installed in the same fashion, was then made, the culture used for inoculating the new series being that tube of the first series which contained the greatest amount of serum in which the bacteria grew well. The transfers were made through many successive generations.

We found that the bacteria cultivated under these conditions lost their faculty of agglutinating in the specific serum, and actually grew more luxuriantly in the later transfer than in ordinary bouillon. This change occurred rather suddenly, usually in the second transfer, never later than in the third. Tables I., II., III. show the result of an experiment performed in this manner.

TABLE I.

July 29, 1903. — To a series of test-tubes containing each 2 cc. of bouillon and 0.3 cc. of a 24-hour bouillon culture of *B. dysenteriæ* (Flexner-Harris) was added anti-dysenteric serum in amounts decreasing from 1.0 cc. to 0.01 cc., and 0.5 per cent salt solution in amounts sufficient to bring the total column of fluid in each test-tube up to 3.3 cc. The series of tubes was put in the thermostat and the results recorded at 24-hour intervals. The serum used in this experiment was anti-dysenteric horse's serum, from Mulford, bottle label 1902, No. 130. Preliminary experiments proved that this serum agglutinated our stock culture in dilutions of between 1 : 200 and 1 : 300.

	Amount of Agglutinin.	July 30.	July 31.	August 1.	August 3.
1...	1.0 cc.	Small, tight clump.	Small, tight clump.	Clump larger and tight.	Clump larger and looser.
2...	0.5 "	Large, tight clump.	Large, tight clump.	" " " "	" " " "
3...	0.25 "	" " "	" " "	" " " "	Clump larger and looser + diffuse growth in fluid.
4...	0.1 "	" " "	" " "	" " " "	Clump larger and looser + diffuse growth in fluid.
5...	0.05 "	Clump breaks up on shaking tube.	" " "	Clump larger, breaks up.	Clump larger and looser + diffuse growth in fluid.
6...	0.025 "	Clump breaks up on shaking tube.	" " "	Clump larger, breaks up; diffuse growth in fluid.	Clump larger and looser + diffuse growth in fluid.
7...	0.01 "	Clumps + diffuse growth in fluid.	Large, tight clump + diffuse growth in fluid.	Clump larger, breaks up; diffuse growth in fluid.	Clump larger and looser + diffuse growth in fluid.
8...	0.0 cc. agglutinin; bouillon control not inoculated.	No growth.	No growth.	No growth.	No growth.
9...	0.0 cc. agglutinin; bouillon + salt solution inoculated.	Abundant, diffuse growth.	Abundant, diffuse growth.	Granular sediment + diffuse growth in fluid.	Granular sediment; abundant, diffuse growth.
10...	0.0 cc. agglutinin; salt solution and bacillus.	No growth.	No growth.	No growth.	Slight growth as sediment.
11...	0.1 cc. agglutinin + salt solution inoculated.	Small clump.	Clump has increased in size.	Clump larger.	Clump larger and looser.

TABLE II.

Aug. 3, 1903.—To a series of test-tubes each containing 2 cc. of bouillon and 0.3 cc. of fluid from tube No. 3, of Table I., was added anti-dysenteric horse's serum (1902, No. 130) in amounts from 1.5 cc. to 0.01 cc. and the tubes filled with salt solution to a volume of 3.0 cc. The results were recorded after 1 and 2 days.

	Amount of Agglutinin.	August 4.	August 5.
1 ..	1.5 cc.	Light, diffuse growth; light, granular sediment.	Abundant, cloudy turbidity in fluid; light, powdery, and finely granular sediment.
2 ..	1.0 "	Abundant, diffuse growth; slight, granular sediment.	Abundant cloud in fluid; powdery sediment — slight.
3 ..	0.5 "	Abundant, diffuse growth; slight, granular sediment.	Abundant cloud; moderate, finely granular, and small flocculent sediment.
4 ..	0.25 "	Abundant, diffuse growth; slight, granular, and powdery sediment.	Moderate cloud; moderate, finely granular sediment with small flocculi.
5 ..	0.1 "	Abundant, diffuse growth; slight, granular, and powdery sediment.	Medium cloud; medium, finely granular sediment; few small flocculi.
6 ..	0.05 "	Abundant, diffuse growth; slight, granular, and powdery sediment.	Medium cloud; moderate, powdery sediment; small flocculi.
7 ..	0.025 "	Abundant, diffuse growth; slight, granular, and powdery sediment.	Medium cloud; moderate, powdery sediment; small flocculi.
8 ..	0.01 "	Abundant, diffuse growth; slight, granular, and powdery sediment.	Medium cloud; moderate, powdery sediment; small flocculi.
9 ..	0.0 cc. agglutinin; bouillon control not inoculated.	No growth.	No growth.
10 ..	0.0 cc. agglutinin; bouillon + salt solution inoculated.	Very abundant, diffuse growth; no sediment.	Abundant cloud; white, moderate, powdery sediment.
11 ..	0.0 cc. agglutinin; salt solution inoculated.	Slight, powdery sediment.	Slight, powdery sediment.
12 ..	0.1 cc. agglutinin + salt solution inoculated.	Abundant, diffuse growth; slight, granular, and powdery sediment.	Abundant, diffuse growth; moderate, powdery sediment with fine grains.

TABLE III.

Aug. 8, 1903.—To a series of test-tubes containing each 2 cc. of bouillon and six drops (0.2 cc.) of fluid from tube No. 1, of Table II., was added anti-dysenteric horse's serum (1902, No. 130) in amounts from 2 cc. to 0.01 cc. and sufficient salt solution to bring the volume in each tube to 3.0 cc. (Tube No. 1, however, contained 4 cc. of fluid.) The results were recorded after 1, 3, and 4 days.

	Amount of Agglutinin.	August 9.	August 11.	August 12.
1..	2.0 cc.	Slight, diffuse, powdery cloud; slight, granular sediment.	Diffuse cloud; slight, flocculent sediment.	Diffuse cloud; slight, flocculent sediment.
2..	1.0 "	Slight, diffuse cloud, not powdery; slight, granular sediment.	Diffuse cloud; slight, flocculent sediment.	Diffuse cloud; slight, ropy sediment.
3..	0.5 "	Abundant, diffuse cloud; slight, granular sediment.	Diffuse cloud; slight, flocculent sediment.	Diffuse cloud; slight, ropy sediment.
4..	0.25 "	Slight, diffuse cloud; slight, granular sediment.	Diffuse cloud; slight, flocculent sediment.	Diffuse cloud; slight, granular sediment.
5..	0.1 "	Slight, diffuse cloud; slight, granular sediment.	Diffuse cloud; slight, flocculent sediment.	Diffuse cloud; slight, granular sediment.
6..	0.05 "	Slight, diffuse cloud; slight, granular sediment.	Diffuse cloud; flocculent sediment.	Diffuse cloud; slight, granular sediment.
7..	0.025 "	Contaminated.	Contaminated.	Contaminated.
8..	0.01 "	Very slight growth; no sediment.	Slight cloud; slight, flocculent sediment.	Diffuse cloud; moderate, granular sediment.
9..	0.0 cc. agglutinin; control bouillon not inoculated.	No growth.	No growth.	No growth.
10..	0.0 cc. agglutinin; bouillon and salt solution inoculated.	Slight, diffuse cloud; no sediment.	Heavy cloud; flocculent sediment.	Diffuse cloud; moderate, granular sediment.
11..	0.1 cc. agglutinin + salt solution inoculated.	Slight, powdery sediment; fluid clear.	Slight cloud; slight, flocculent sediment.	Fluid clear; slight, ropy sediment.

This experiment was repeated a number of times, using different supplies of bouillon, different stocks of anti-dysenteric serum, different stock cultures of the Flexner-Harris bacillus, as well as a stock of the alkaline type of B. dysenteriæ. The slight irregularities observable in the tables disappeared as the technic was perfected, and the series ran with great uniformity.

The bacteria will hardly grow at first in the tube containing twenty-five per cent (one-half cubic centimeter) or more of serum, and the growth, after several days, settles at the bottom of the test-tube in flakes having much the consistency of wet filter paper. In the later transfers, on the contrary, the bacteria grow luxuriantly, even in those tubes containing fifty per cent or more of serum, forming a turbid cloud throughout the fluid, and having a sediment with clumps not larger than those found in the bouillon control, which is free from serum. The changed bacteria regularly grow in fluid media — even in serum-free media — as a diffuse cloud consisting of single rods, great numbers of long threads, and considerable numbers of small granular clumps.

In the later transfers of one series the culture died out entirely, and in another series the growth was greatly retarded in a later transfer. Thus the fourth transfer in Series II. showed no growth in any of the tubes before the end of the third day, but after several days the culture seemed to regain its vitality and grew with the usual luxuriance.

We have carried the bacteria through more than twenty transfers of serum, and have not been able to observe any change in the agglutinability after the third transfer. We could not note the progressive change described by Walker. It seems to make little or no difference whether the transfers are made every twenty-four hours or at longer intervals. We have also tried making transfers at very short intervals, and find that the bacteria can be made to lose their agglutinability within a few hours by making frequent transfers at short intervals. Furthermore, there were indications that the change in the bacteria was the same whether they were grown in concentrated or diluted horse's serum. We did not try the great dilutions used by Kirstein.

The controls that were made at each transfer showed some features of interest. The litmus milk, mannite and litmus-glucose-agar controls showed no variations. The cover-slip and hanging drop preparations showed from the outset many degeneration forms, and after the second transfer the bacteria appeared in increasing numbers in the form of very long, branching threads, presenting a marked contrast to the short blunt rods of the stock culture. This thread-like growth has been described by Landsteiner,[7] Pfaundler,[11] Bail[1] and others. The growth on the agar plates frequently became more luxuriant after the transfer into anti-dysenteric serum. The size of the agar colonies increased slowly until individual colonies in some series measured six to eight millimeters in diameter. The characteristic odor of the colonies on agar plates remained unchanged. The growth in bouillon was much more luxuriant after one or two transfers from serum to serum.

Several experiments were performed to determine the permanency of the change brought about in bacteria by this process of "immunization." Immunized bacteria were transferred to bouillon and the new culture tested for its agglutinability after eighteen to twenty-four hours. In other cases the immunized bacteria were transferred to agar slants, thence to bouillon and tested after two or three days of transplanting. The bouillon transfers usually showed a decided loss in the "immunity." The agar transfers also showed a loss. After two or three such transfers the bacteria had returned almost to their original condition. However, the bacteria exhibit considerable variations in this respect, the immunity in some cases disappearing very quickly; in other cases lasting through several transfers. We did not try to determine the basis for these variations in the persistency of the change. This question has been worked upon by Müller, Walker, Kirstein and others, and so far no permanent specific change has been produced, though one or two authors think it possible to make such a change permanent by continuing the transfers for a long period. In the experiments of Park and Collins the recovery of agglutinability was slow, requiring many weeks of transferring from agar to agar.

We are evidently dealing here with a process of adaptation, which resembles a form of immunization, whatever the controlling mechanism may be; the end result being that a bacterium is enabled to grow most luxuriantly in a medium originally unfavorable to the bacterial species, and that an agglutinable species is converted into a nonagglutinable species.

The theoretical explanation of the mechanism upon which this "immunity" depends is important. At the present time we know of two forms of active, acquired immunity. In the first place, it is the usual thing for an immunized animal to develop new substances or antibodies, which protect against the material inoculated. In a few cases, however, a different type of immunization has been observed. In this second type the animal loses those receptors which have affinity for the inoculated material. This type of reaction was found by Ehrlich[3] and discussed quite fully in the article dealing with the isolysins. It has also been observed that the red blood corpuscles of rabbits immunized against eel serum lose, in part, the receptors with affinity for the amboceptors of eel serum. The "natural," or "absolute," or "racial" immunity of some animals for certain bacteria and toxins is also due to lack of susceptible receptors. We must, then, determine whether the adaptation noted in our experiments conforms to either of these types of immunity.

If the immunity of the bacteria is due to protective antibodies which are formed by the bacteria and set free in the surrounding serum containing culture fluid, the protection afforded must be limited, and the addition of an excess of serum will still agglutinate the bacteria as well after immunization as before. Also, if only that amount of serum is added which does not agglutinate the bacteria, the agglutination must fail, because the protective substances set free by the bacteria have united with the agglutinins of the serum. If such a union occurs, this serum should not subsequently be capable of agglutinating the stock cultures of bacteria.

On the other hand, if the immunity of the bacteria is due to the loss of the susceptible receptors, the immunity may be absolute, if all of the susceptible receptors are lost; and furthermore, the growth of the bacteria in the agglutinating serum should not diminish the agglutinating properties of the serum.

We tested this question in the following way: A test-tube containing two cubic centimeters of bouillon and 0.25 cubic centimeter of anti-dysenteric horse's serum was inoculated with a fresh stock culture of the dysentery bacillus of the acid or Flexner-Harris type. Transfers were made every day into a fresh tube of bouillon anti-dysenteric serum, and a nonagglutinating type of bacillus was developed. The eleventh transfer was allowed to grow in the thermostat as usual, until the fluid became diffusely turbid (eighteen to twenty-four hours). The tube was then centrifugalized, the supernatant fluid was drawn off with a pipette, the sediment washed with normal salt solution, centrifugalized and again suspended in salt solution. The following tests were then made simultaneously:

(1.) A hanging drop preparation from this suspension showed: (*a*) great numbers of free individual bacteria with active Brownian movements; (*b*) a number of bacteria growing in long threads; and (*c*) a moderate number of small granular clumps of bacteria. The controls show that the changed bacteria grow in plain bouillon in the same way. Hanging drop agglutination tests were made with this suspension and the stock anti-dysenteric horse's serum, in dilutions varying from 1: 20 up to 1: 1000. No clumping occurred in these preparations, the small granular clumps which were found in all preparations being identical in appearance with those found in the control, the numbers of free single bacteria with active Brownian movements and the thread forms remaining unaltered. After a few hours the multiplication of the bacteria was very marked, particularly in the concentrated serum (1: 20) and least in the control and in the more diluted serum preparations. After two days a few large clumps were found in the 1: 20 preparation.

(2.) At the same time a control agglutination test was made with the same serum, and the normal stock culture of dysentery bacillus. After the hanging drops had stood for six hours there was definite clumping in the stronger dilutions, and in 1: 500 and 1: 1000 there were small clumps, almost no single bacteria and no Brownian movements, in dilutions of 1: 1500 the clumps were still suggestive.

(3.) At the same time the supernatant fluid from which the bacteria had been centrifugalized was tested for its power to agglutinate the stock culture of dysentery bacillus. Large, loose clumps occurred as perfectly as with the control serum. In fact we were impressed by finding agglutination in dilutions of 1 : 2000 of this centrifugalized serum.

The experiments showed: 1st. That the immunized bacteria had not neutralized nor removed any agglutinins from the serum, demonstrable within the limits obtainable by our technic, the serum still agglutinating the stock culture as completely as in the controls; 2d. The bacteria washed with salt solution to remove antibodies showed no tendency to agglutinate in the stock serum; 3d. After a long while (two to three days) some bacteria which have grown in the concentrated serum-bouillon will agglutinate; 4th. Growth occurs faster in the serum than in plain bouillon; 5th. Not all the bacilli in a nonagglutinating culture fail to agglutinate.

These results can be interpreted in only one way. They show that the bacilli have not formed any protective antibodies, but have protected themselves by losing their susceptibility to the agglutinins.

In the terms of the receptor theory we must say that these bacteria have no longer available those receptors capable of uniting with the agglutinins. In other words the bacteria have apparently acquired an "absolute" or "racial" type of immunity for the agglutinins occurring in the serum of a horse previously immunized against this species of bacterium.

We must note here the conflict between our results and those of Walker. From his experiments Walker concluded that the typhoid bacilli reacted to the specific antityphoid serum, forming antiagglutinins, and he found that growing his modified bacilli in the serum caused a progressive diminution in the power of the serum to agglutinate the stock culture. Our results are in accord with those of Bail and others on this point.

The question of the specificity of this reaction was next taken up. If several animals of species A are immunized with red blood corpuscles from different members of species B and from a closely related variety, B′, we expect the serum of each immunized animal to be hemolytic for any member of species B, but we also expect to find slight, quantitative, individual differences in the action of the serum and corpuscles from the various experimental animals. These differences would be due partly to individual differences in the receptors possessed by the different members of species A, and partly to individual differences in the red corpuscles of different members of species B, and especially to the differences between blood from B and B′. If the immunity of the bacteria with which we are dealing is specific, we can expect to find the bacilli most completely protected from the action of the serum with which they were immunized, but showing quantitative differences in their immunity to serum of other horses inoculated with the same or with other stocks of dysentery bacillus. Our experiments fail to show such a condition. We had at our disposal serum from two horses immunized against different stocks of the "acid" or "Flexner-Harris" type of dysentery bacillus; serum from one horse immunized against the non-acid type, and serum from one normal horse. We found that our bacilli immunized by growth in serum from one immunized horse acquired the same type of immunity for each one of these different specific and normal sera, while the stock culture showed a certain degree of susceptibility to each of the sera.

In this series of experiments, serum, specific for the "Flexner-Harris" type of dysentery bacillus, agglutinated our stock culture in dilutions up to one to six hundred. This serum failed to agglutinate our "immunized" bacteria in dilutions of one to twenty, though clumps were found in the one to twenty dilution after two days.

Horse's serum, specific for the non-acid or Shiga type of dysentery bacillus, agglutinated our stock culture (Flexner-Harris or acid type) in dilutions up to one to five hundred. This serum failed to agglutinate our "immunized" bacillus in a dilution of one to twenty, though large clumps formed in the one to twenty dilution at the end of two days.

Normal horse's serum agglutinated the stock culture of dysentery bacillus in dilutions of one to one hundred, and caused small clumps to form in dilutions of one to two hundred. Normal horse's serum fails to agglutinate the "immunized" dysentery bacillus in one to twenty dilution.

This result is hardly what one should expect from experiments on animals. It seems that this change in the bacteria is much more far reaching than the changes occurring in the higher animals.

We then tested the specificity of the reaction still further, by reversing the above procedures. Bacteria from the original stock were grown in bouillon plus normal horse's serum through several generations. As in the other cases, the bacteria rapidly lost their agglutinability to the normal serum. These cultures, however, differed from those grown in specific serum, in that the growth seemed less active and less luxuriant in the normal serum. After ten transfers these bacteria were tested with the specific agglutinating serum and the surprising result was obtained that the bacteria had almost altogether lost their power to agglutinate with the specific serum, which gave only a trace of agglutination in a one to fifty dilution after standing for twenty-four hours. If one adopts that hypothesis which supposes that the serum of the immunized horse contains, in addition to its normal constituents, a number of new substances set free in the serum as the specific result of immunization, it is hard to understand by any stretch of the receptor theory how bacteria grown in normal serum, in which these specific substances do not exist, can be rendered immune to serum containing these specific substances. The work of Park and Collins[10] (p. 492) may explain this in part. It was noticed that whereas the immunized bacteria do not pick up any agglutinins from the specific horse's serum, bacteria immunized in normal serum appear to destroy the agglutinins of the normal serum almost altogether. After the immunized bacteria had been grown in normal serum for eighteen hours, this serum failed to agglutinate the stock culture of bacteria, even in dilutions of one to fifty.

A series of experiments was then arranged to test the specificity of this immunity still further.

Parallel series of sub-cultures from the stock dysentery bacillus were made: 1, upon bouillon plus human serum; 2, upon bouillon plus pleuritic fluid; 3, upon bouillon plus dog's serum; 4, upon bouillon plus chicken's serum; 5, upon bouillon plus anti-dysenteric horse's serum which had been passed through a Berkefeld filter; 6, upon bouillon plus anti-dysenteric horse's serum which had been heated for forty minutes at 60° C. After these series had been carried through from six to nine transfers, the last sub-culture was centrifugalized, the sediment consisting of bacteria was washed, suspended in salt solution, and its agglutinability tested with normal horse's serum and with the anti-dysenteric horse's serum. The results are tabulated below.

TABLE IV.

B. Dysenteriæ modified by cultivation for several transfers in human serum, pleuritic exudate, etc., respectively; the final transfer centrifugalized after eighteen hours' growth, the bacteria suspended in salt solution and tested for agglutinability with normal horse's serum.

Normal horse's serum.	B. dysenteriæ modified by cultivation in human serum.	B. dysenteriæ modified by cultivation in pleuritic exudate.	B. dysenteriæ modified by cultivation in dog's serum.	B. dysenteriæ modified by cultivation in chicken's serum.	B. dysenteriæ modified by cultivation in filtered horse's serum.	B. dysenteriæ modified by cultivation in heated horse's serum.	Stock bouillon culture of B. dysenteriæ; eighteen hours.
1 : 40.	Clumps slightly larger than in control.	Large loose clumps.	Possibly more clumps than in control.	Like control.	Like control.	Like control.	Strong agglutination.
1 : 200.	Like control.	Large loose clumps.	Like control.	Like control.	Like control.	Like control.	Suggestive agglutination.
0.0 cc. normal horse's serum. Control of bacteria alone.	Small clumps and free bacteria.	Small clumps and free bacteria.	A few clumps; many free bacteria.	A few clumps; many free bacteria.	1–2 small clumps; many free bacteria.	A few small clumps and threads of many free bacteria.	No agglutination; short free rods.

TABLE V.

B. Dysenteriæ modified by cultivation for several transfers in human serum, pleuritic exudate, etc., the final transfer being centrifugalized after eighteen hours' growth, the bacteria suspended in salt solution and tested for agglutinability with specific anti-dysenteric horse's serum.

Anti-dysenteric horse's serum diluted.	B. dysenteriæ modified by cultivation in human serum.	B. dysenteriæ modified by cultivation in pleuritic exudate.	B. dysenteriæ modified by cultivation in dog's serum.	B. dysenteriæ modified by cultivation in chicken's serum.	B. dysenteriæ modified by cultivation in filtered horse's anti-dysenteric serum.	B. dysenteriæ modified by cultivation in anti-dysenteric horse's serum heated to 60° for forty minutes.	Stock culture of normal B. dysenteriæ; fresh transfer.
1:40.	Definite agglutination.	Large, loose clumps.	Definite agglutination	No agglutination.	No agglutination.	No agglutination; thread growth.	Strong agglutination.
1:200.	Suggestive agglutination.	Suggestive clumping.	Definite agglutination.	No agglutination.	No agglutination.	No agglutination.	Definite agglutination.
1:400.	Very doubtful.	Like control.	Decreasing agglutination.	No agglutination.	No agglutination.	No agglutination.	Definite agglutination.
1:800.	Like control.	Like control.	Decreasing agglutination; still positive.	No agglutination.	No agglutination.	No agglutination.	Decreasing agglutination.
0.0 serum. Bacterial suspension alone.	Small clumps and free bacteria.	Small clumps and free bacteria.	Few clumps; many free bacteria.	Few clumps; many free bacteria.	One or two small clumps; many free bacteria.	A few clumps and threads; many free bacteria.	Many short blunt rods.

In this experiment it is seen that the bacteria grown in these various media have their agglutinability to a specific serum modified considerably. The change is most marked in bacteria grown on chicken serum and on the specific agglutinating horse's serum which had been previously heated or passed through a Berkefeld filter; least marked in bacteria grown on pleuritic fluid. The change in agglutinability is shown toward both normal horse's serum and specific, agglutinating horse's serum.

We had not an opportunity to repeat the last series of experiments nor to determine accurately the quantitative differences produced by the various agents. However, it is clear that this loss of agglutinability, when bacteria are grown upon the serum, is not a specific reaction either in a chemical sense, or in the biological sense, as usually meant. It is to be noted, however, that the bacteria cultivated on specific anti-dysenteric serum gave the most striking results.

If we summarize our results, we may say that:

(1.) Bacillus dysenteriæ, grown in the presence of specific agglutinating horse's serum, loses its susceptibility to the agglutinins in the specific antidysenteric serum used in the culture medium, to the agglutinins in normal horse's serum, and to the agglutinins in the serum of other horses immunized against the same stock culture of dysentery bacillus or against other varieties of dysentery bacillus.

(2.) Bacillus dysenteriæ grown in the presence of normal horse's serum loses its susceptibility to agglutinins in the same manner and apparently to the same degree as if grown in the presence of anti-dysenteric horse's serum.

(3.) The modified bacteria have not the power to unite with the agglutinins in normal or specific horse's serum.

(4.) The modified bacteria appear to injure in their growth the agglutinins in normal horse's serum, but they may be grown in anti-dysenteric horse's serum for at least twelve to eighteen hours without impairing the power of the serum to agglutinate a stock culture of dysentery bacillus.

(5.) The absence of agglutinability is not a permanent characteristic of the modified bacteria, but disappears more or less rapidly when the bacteria are grown on media free from serum.

(6.) Bacillus dysenteriæ in the presence of anti-dysenteric horse's serum grows more luxuriantly, the growth being characterized by the presence of long branching threads and of small granular clumps, which appear even after transfers are made upon bouillon.

NOTE. — We are not convinced that the Pfaundler's threads represent an end to end agglutination, as other writers suggest. We find these threads in non-agglutinating cultures and in cultures which will not unite with agglutinin, and we also find them growing in the serum-free bouillon cultures of our nonagglutinating variants. We are therefore inclined to look upon the thread reaction as something independent of the phenomenon of agglutination.

(7.) The agglutinability of Bacillus dysenteriæ can be affected in the same way by some other media as it is by anti-dysenteric horse's serum. For example: the bacillus becomes non-agglutinable or less agglutinable for anti-dysenteric horse's serum and for normal horse's serum if it is grown in the presence of human serum, dog's serum, chicken's serum, or of anti-dysenteric horse's serum previously heated to 60° C. or passed through a Berkefeld filter.

That the characteristics of bacteria can undergo alteration is well known. Alterations in virulence, in the power to form pigments and toxins, in motility, and in vitality are constantly observed in laboratory cultures, and in bacteria obtained at autopsy and from experimental animals. Bancel, Müller,[9] Sacquépeé, McWeeney,[8] Courmont, and Nicolle et Thenel have reported finding in typhoid patients strains of B. typhosus, characteristic culturally but failing more or less completely to agglutinate. In Bancel's cases the nonagglutinability persisted for from six to eleven months in the aboratory cultures. Usually the bacteria regained their power to agglutinate after being transferred a few times upon artificial culture media.

Pasteur was the first to alter bacteria experimentally. By inoculating susceptible animals he was able to increase the virulence and vitality of bacteria, and by other methods, such as drying, he could diminish the virulence. Haffkine in 1890 educated bacteria to grow in aqueous humor, a fluid normally injurious to the bacteria, while Roger, on the contrary, decreased the virulence of streptococcus by using immune serum in the culture media. Ainley Walker,[14] Ransom and Kitashima, Bail,[1] Muller,[9] Trommsdorff,[13] Park,[10] and Kirstein,[5] obtained results like those of Haffkine. Walker found that typhoid bacilli grown in bouillon containing immune serum lost their power to agglutinate, became more virulent, developed an increased power to resist antityphoid serum, and produced anti-agglutinins. This last finding is not confirmed by other workers.

Bail's investigations of the changes produced in bacteria and agglutinin are very important. His results may be given briefly.

> Typhoid bacilli injected into the peritoneal cavity of a guinea-pig lose their agglutinability. The loss of agglutinability is not complete, and not all the bacteria are affected. The change comes suddenly, with little warning, about three hours after inoculation. The inagglutinable bacteria cannot unite with the agglutinins in specific serum. Heating the modified bacteria to 60° C. renders them again at least slightly agglutinable.
>
> He could not render cholera vibrios inagglutinable by injecting them into the peritoneal cavity of a guinea-pig.
>
> He rendered typhoid bacilli inagglutinable by holding them for a short while (one to three hours) in dilutions of immune serum from which agglutinins had been removed by previous treatment with an excess of agglutinating bacteria, or by holding them in dilutions of immune serum in which the agglutinins had been destroyed by heating to 75°.
>
> Animals inoculated with his modified bacilli produced an unusually powerful agglutinating serum. This serum was more active than ordinary immune serum in agglutinating both his modified bacilli and also ordinary stock cultures.

Bail is not content to explain these phenomena as the result of alterations in the susceptibility of the bacteria to agglutinins, and thinks also that they cannot be explained as the result of the presence of agglutinoids. In order to explain them he assumes that the agglutinin has a structure, not such as Ehrlich describes, but like that of a hemolysin. The portion corresponding to the amboceptor he calls "agglutinophor," the complement he calls "hemiagglutinin." He thinks that the bacteria which he modified were inagglutinable because of the presence of agglutinophore without hemiagglutinin.

In the last part of his paper Bail describes numerous experiments, complicated and frequently ambiguous, designed to demonstrate hemiagglutinin. His experiments are ingenious and a few of them may be interpreted as supporting his hypothesis, but upon the face of them they are not convincing.

Bail's assumption that bacteria become nonagglutinable as the result of having something added to them from the agglutinating fluid fails to explain the following facts: (1) fresh stock bacilli are agglutinated by this serum; (2) the nonagglutinating tendency of the modified bacteria is carried over through several generations; (3) in almost every agglutinating reaction a few bacteria escape agglutination.

Bail thinks that he has altered the individual bacteria by his treatment, but this is at least open to doubt. As we suggest elsewhere, it is possible to explain these reactions upon the assumption that the agglutinating bacteria inoculated into his test-tubes disappeared or lay dormant for a while, while the nonagglutinating variants multiplied rapidly under what is evidently a stimulating influence exercised by the immune serum. This view gains support from his animal experiments, where lysins and bacteriotoxins remove fairly completely the non-resistant individuals from the culture; whereas, in the test-tube experiments the modification evidenced is only the average between the modified and not modified bacteria.

Even if Bail is justified by his partly successful experiments in suggesting the hypothesis of the presence of agglutinophors and hemiagglutinins, this hypothesis failed to explain the hereditary character of the inagglutinability as it is met with in typhoid bacteria cultivated from human bodies, and as it has been found by Walker, Park, Kirstein, and others, and in these test-tube experiments. In short, it seems to us that Bail has failed to recognize the prime importance of the bacteria themselves as the bodies reacting in this phenomenon.

Muller[9] adopted a technic somewhat different from Bail's. He proved conclusively that the nonagglutinability is not due to the presence of agglutinophores, nor is it due merely to the use of a concentrated serum. The bacteria did not form antiagglutinins, but for several generations the modified bacteria preserved the power to resist the action of agglutinating serum.

Park and Collins,[10] while studying the specific and group agglutinins that can be produced by inoculating animals with various dysentery and colon bacilli, found that the stock cultures of dysentery bacillus became inagglutinable when grown for eleven transfers in fifteen per cent immune serum, and ceased to absorb its specific agglutinins. The recovery of agglutinability was very slow, and its development gradual in the modified stock, weeks or months of transferring from agar to agar being required before the bacteria agglutinated as readily as the parent stock.

Experiments indicate quite clearly that bacteria may be affected in more than one way by their environment. It is very clear that bacteria grown in agglutinating or nonagglutinating serum lose their property of uniting with agglutinin. In other words, the haptophore group of the agglutinable substance (Eisenberg and Volk[4]) is the factor which is interfered with in some way. This group is not destroyed, for these bacteria produce strong agglutinins. Also the bacteria grown in immune serum grow more luxuriantly and are more virulent than the stock culture. This result is not constant, however. Roger decreased the virulence of Streptococcus erysipelatos by cultivation in immune serum, and Charrin and Roger thought that immune serum decreased the virulence of B. pyocyaneus. Pfeiffer and Friedberger found that virulent cholera vibrios will unite with more amboceptors in immune serum than non-virulent vibrios can unite with.

Kirstein attempted to modify bacteria in various ways. He confirmed the work of Eisenberg and Volk, demonstrating the binding and functioning group in the agglutinable substance. He found that the binding group is very resistant, withstanding dessication, treatment with alcohol, and pulverization. He was able to lower the agglutinability of bacteria by growth in serum. He found that B. prodigiosus, grown at 37° C., had almost completely lost its power to agglutinate, but that this bacillus could still produce a powerful agglutinating serum. He did not determine whether this modified bacillus could unite with agglutinin or not. He had very little success in modifying the agglutinability of typhoid bacteria by his experimental methods. He was able to produce spontaneous agglutination by growing B. typhosus in media free from albumin. This property of spontaneous agglutination was not studied more closely.

The whole subject of the variations and modifications occurring in bacteria is ably reviewed by Deutsch and Feistmantel.[22] They describe the natural and artificial modes of increasing and decreasing the virulence of bacteria, and review the experiments in which the virulence of a bacterial stock has been altered for one species of animal without altering its virulence for other species of animals. They are of the opinion that "the virulence of a bacterium, according to our present ideas, must be regarded as the expression of the degree of immunity of the bacterium toward a susceptible animal body."

When we note the wide differences between these reactions and those occurring during the process of immunization in animals, we are disposed to question the correctness of regarding the change in the bacteria as a process of immunization at all in the usual sense of the word. Furthermore, we are not by any means convinced that we have modified any individual bacterium which was originally agglutinable. On the contrary, while recognizing that this is an open question, we are disposed to adopt temporarily a different hypothesis. We suspect that the conditions of our experiments are particularly favorable to the multiplication of those individual dysentery bacilli in our cultures which are nonagglutinable variants, and that these variants rapidly outgrow the ordinary agglutinating bacilli, which multiply very little, if at all, during the first few hours after agglutination occurs, and that our nonagglutinating cultures are merely descendants of these variants. Whether the small clumps found after modifying the bacteria are descendants of modified bacteria, or arise from agglutinable bacteria which have been carried over from one culture to the next, cannot be determined by the technic employed. The clumps in the later transfers could be reduced in number if the inoculation was made from the surface of the fluid, so as to include as little sediment as possible.

Although we can modify the bacteria in such a way that they will not unite with the specific agglutinin, we are altogether unable to explain the mechanism of this process. We cannot even say whether the bacteria which fail to agglutinate differ from the bacteria of the stock culture by the absence of those chemical groups which unite with agglutinin, or whether the difference between these bacterial strains is more of a physical than a chemical phenomenon; or whether the bacteria are protected against the agglutinins by some intrinsic anti-substance in the bacteria, in a manner analogous to the physiological blocking of the rennet in the circulation by an anti-rennet. It is of interest in this connection that Bail could partly restore the agglutinability of modified bacteria by heating them to 60° C.

We think it safer, under the circumstances, not to regard the reactions as true reactions of immunization, but as processes of adaptation and selection.

It appears at first sight that we have here evidence bearing closely upon the question of the transmission of acquired characteristics. If the transfers from the immunized bacteria are still immunized, it would seem to be a case in which the acquired immunity has been transmitted. However, it is equally easy to explain this phenomenon as due to the selection and adaptation of individual bacteria with little or no susceptibility to the specific agglutinins of horse's serum, and this seems to be the more probable explanation. If a careful search be made through a hanging drop showing even the most typical agglutination, one or two bacteria can often be found which are more or less unaffected by the agglutinins. We consider it probable that these one or two bacteria give rise to the nonagglutinating variants which we have developed.

We cannot agree with Walker in thinking that we have to deal with bacteria which, as individuals, have acquired a new characteristic and handed it on to be intensified in their descendants, and we think it more probable that the process of adaptation and development of variants affords as satisfactory an explanation of our results as it does of other botanical or zoölogical variations. At the same time Walker shows clearly why we do not get the very marked results with our method that is obtained by the method of passage, for in this latter case only the most resistant bacteria survive, the others succumbing to the bacteriotoxins; while in our test-tube experiments, there is no bacteriotoxic action to speak of, and our variant represents only the average change of the bacteria. This view is further supported by slight variations observed from time to time in our transfers. The bacteria grown in bouillon plus antidysenteric serum grow at first very slowly and as a thick felt-work growth at the base of the test-tube. After one or two transfers the felt-work growth no longer forms, but the bacteria grow as fine, powdery or granular clumps, some of which occur as a precipitate in the test-tube, others being suspended in the fluid. A slight disturbance will spread the precipitate through the fluid. After a few transfers, the fluid tends to become more diffusely turbid and the number of granular clumps diminishes. If, now, we make our transfers from the layer of fluid that was more diffusely turbid, the new sub-culture shows even fewer granules, but if we happen to carry over many of the clumps into the new sub-culture this shows also numerous clumping bacteria.

The phenomena described above, and in the literature referred to, have an important bearing on the question of the clinical significance that should be attached to the reaction of agglutination. Unfortunately, it seems that we can make no more positive statement at present than to say that the absence of the reaction of agglutination is not of specific diagnostic importance, but the occurrence of this reaction, under proper experimental conditions, is specific.

This one example raised a doubt as to the fundamental character of the other supposedly specific differences, based only upon function between the bacilli of this group (the colon-typhoid group).

We do not think that we have developed a new species, but look upon the modified cultures as variants.

We see no reason to think that the presence of the reaction of agglutination is any less significant in B. dysenteriæ than is the same reaction in other bacteria. The frequency with which the reaction is absent has produced an impression that the agglutination of B. dysenteriæ is of no significance, a view which we cannot accept.

It may be said that the results which we have obtained are simply the old and well-known results obtained by growing bacteria under unfavorable conditions. The obvious reply is, that the nature of the change produced by unfavorable conditions is not explained by this criticism. Here we have one definite condition introduced into the environment of the bacteria. The changes resulting from this condition have been studied. What changes result from other unfavorable conditions remains unsettled. We may reply, furthermore, that we actually have not introduced an unfavorable condition; but, on the contrary, the bacteria grown in the specific serum show a definite increase in the luxuriance of their growth. Furthermore, Walker found very definitely that the effect of growing typhoid bacilli in anti-typhoid serum was quite the reverse of unfavorable; for his modified bacteria not only were inhibited to a less degree by the anti-typhoid serum, but they also proved more virulent on inoculation into guinea-pigs; in other words, their vitality was increased by the treatment.

These experiments call attention to a new way of increasing the virulence of bacteria. By growing the bacteria in serum, we have evidently lessened the susceptibility of the bacteria to those anti-bodies which protect the animal against the bacteria; that is to say, we have increased the resistance of the bacteria. This increase in the resistance of the bacteria amounts actually to an increase in the virulence of the bacteria, because their chances of surviving are increased.

It is plain that this mode of reaction among bacteria may have an important bearing upon the rise and spread of epidemics. Most pathogenic bacteria find conditions unfavorable to their multiplication when they are outside of their host; and further, most hosts, even though susceptible, can successfully combat infection with a small number of pathogenic bacteria. But if these bacteria had developed a peculiarity which enabled them either to prolong their existence outside the body or to resist the normally destructive action of the body, then the chances for the perpetuation of this strain of bacteria would be increased, probably enormously, and such a bacterial strain might readily give rise to an epidemic.

It seems reasonable to suppose that the reactions occurring in these test-tube experiments are identical with those taking place during the growth of bacteria in the infected animal body; except that in the latter case the bacteriotoxins and other protective mechanisms of the diseased animal as well as agglutinins are acting upon the bacteria. The infecting bacteria are not agglutinated, although they are surrounded with an abundance of agglutinating substance, nor can the bacteria in the fluids and exudates of the infected animal be agglutinated by the addition of extraneous agglutinating sera. Here, as in the test-tube experiments, we may assume either that something added to the bacteria from the surrounding fluids of the animal prevents agglutination from occurring, or that some form of immunization takes place in individual bacteria, or that the surviving bacteria in an infected animal are descendants of only those bacteria which possess, to start with, a natural insusceptibility to the antibacterial mechanism of the infected body. This remains an open question, though, as stated above, we are inclined to look upon the third explanation as the most satisfactory, and we are inclined to look upon the bacteria producing a fatal septicemia in a moderately resistant animal as hardier variants from the ordinary stock. Be that as it may, our experiments do not determine the real nature of the protection or modification. It is altogether unsettled what brings about the apparent loss of agglutinable substance in the bacteria. Whether it is due to an alteration in the surrounding membrane, whether a capsule veils the agglutinable substance, or whether some anti-agglutinable substance of bacterial origin lacks the bacterial receptor for agglutinin, after the manner in which rennet and anti-rennet are united in the body, is at present a matter of speculation.

The form of reaction by the bacteria which these experiments demonstrate is after all not so remarkable if one thinks of the conditions under which pathogenic bacteria live. They grow usually upon or in the presence of some living organism, which must be many times the size of the bacteria. The larger organism is capable of producing much larger amounts of protective anti-bodies than the minute short-lived bacterium. If the bacteria protect themselves by anti-body formation, it can hardly be supposed that they can compete successfully with the hosts in which they reside; whereas, by the simple mechanism of losing or of veiling their susceptible receptors, the bacteria are at once placed upon a basis where they can compete, with a fair show of success, against the anti-body-producing host. It would be interesting to determine whether the type of reaction described in this article is of common occurrence in the vegetable kingdom.

When one considers the facts presented in this article and in the literature referred to, when one thinks of the great number of species and varieties of bacteria nearly related, such an example, for instance, as is afforded by the colon-typhoid group, it does not seem to be wildly speculative to suppose that bacteria have their fixed characteristics reduced to the simplest biological requirements, that their adaptability is very great, so that they are enabled to survive under quite a wide range of conditions, and that there are many forces, of which the experiment utilizes one, which will call forth changes in the bacteria even more far-reaching than those recorded above. We may even go so far as to suppose that a bacterial species which is non-pathogenic may, under suitable conditions, give rise to a pathogenic strain, and we may expect to find new pathogenic varieties arising from time to time, through the agency of variation and selection.

[We take pleasure in thanking Drs. Emerson and Cole of the Johns Hopkins Hospital for many courtesies throughout our work. We also wish to express our indebtedness to Dr. Kinyoun, who kindly furnished us with serum from the Mulford establishment.]

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THE CHEMISTRY OF ATHEROMA AND CALCIFICATION (AORTA).*

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This investigation owes its inception to an attempt to study by micro-chemical methods the sequence of the changes occurring in the experimental lesions produced in the rabbit's aorta by the administration of adrenalin. As, however, micro-chemical methods proved unsatisfactory and analytical methods impossible, on account of the small amount of substance in the rabbit's aorta, the work as originally planned was abandoned in favor of a study by analytical methods of the chemistry of the various stages of arteriosclerosis in the aorta of man. The studies of Klotz,[1] which appeared shortly after the beginning of the work, and those of Wells,[2] which were published previous to its conclusion, are so recent that they render a general survey of the literature of calcification unnecessary.

As this work has to do mainly with the relation of the fatty acids and lecithin[3] (phosphorus) to calcification, the results of Klotz and Wells have a very important relation and the views of these investigators may be briefly stated at this time. Klotz employing chiefly micro-chemical methods believes he has demonstrated that the fatty acids play an important part in calcification. In a few qualitative examinations of calcified material, he found what appeared to be calcium soaps. These observations he attempts to support by micro-chemical studies which, however, are not convincing. His demonstration of soaps depends on a peculiar staining with Sudan III., of calcium — principally upon a black discoloration with an aqueous solution of silver nitrate (Kossa's stain). The occurrence in close contact of granules staining by each of these methods is taken as evidence

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of the presence of calcium soaps and the conclusion is drawn that the formation of these soaps is the first step in the process of calcification. The inaccuracy of this method of investigation has been pointed out by Wells, who, using purely analytical methods, reaches a different conclusion. He finds there is no essential difference between the processes of normal ossification and pathological calcification. No evidence was obtained to indicate that calcium soaps form a constant and important stage in the process of calcification. That they may be present in small amounts is not denied. His experimental studies also speak strongly against phosphoric acid, arising from decomposed nucleo-proteids, as an important binder of calcium.

The investigations here to be described deal with the study of nine aortas. Of these three were normal, two were aortas with extensive atheroma but no macroscopic evidence of calcification, and four showed various grades of calcification. One of these latter was the seat also of large "atheromatous ulcers." As several of the points studied have been covered by Wells' recent publication, these results will be stated but briefly.

The preparation of the material was as follows: The entire aorta from heart to division of the iliacs was removed and the intima and media carefully separated from the adventitia in order to exclude all extraneous fat. The material was now cut into small pieces, dried in absolute alcohol over a water bath, ground to a powder, and then placed in a flask with reflex condenser and boiled with absolute alcohol for periods varying from twenty-two to fifty-four hours and afterwards extracted in the Soxhlet apparatus for from nine to thirty-one hours. To facilitate extraction, the material was reground every twelve to fifteen hours and fresh alcohol added. The powdered residue was now extracted in boiling chloroform twenty-two to thirty-four hours and in the Soxhlet apparatus seven to fourteen hours. The use of ethyl ether, which was one of the solvents first employed, had to be discontinued on account of the fact that numerous specimens reacted acid to phenolphthalein. Since this indicator served in the determination of the fatty acids, a neutral solvent became extremely essential. The extractive material taken up in absolute alcohol, boiling absolute alcohol, and chloroform was in each case entirely soluble in these solvents. An analysis of the extract was then made for calcium soaps, fatty acids, and lecithin as follows: To be absolutely certain of the solubility of calcium soaps, calcium stearate was prepared artificially. It was found to be sparingly soluble in cold absolute ethyl alcohol, considerably soluble in hot absolute ethyl alcohol, but insoluble in chloroform. According to Wells, "calcium oleate is less soluble in ether than in alcohol and the palmitate and stearate dissolve very little in ether, although considerably in ethyl alcohol; all dissolve much better in amyl alcohol, probably largely because of its high boiling point." According to the method of extraction, the calcium soaps should become soluble in the absolute alcohol and appear in the extractive material. The extracts were tested for calcium by fusing with sodium hydrate and potassium nitrate, precipitating with ammonium oxalate and weighing as CaO. The fatty acids were titrated in alcoholic solution with alcoholic $\frac{N}{10}$NaOH using phenolphthalein as indicator.

Since the phosphorus contained in the alcohol-chloroform extracts was wholly organic in character, it could be considered that the P-content depended upon the presence of some form of lecithin.[4] The P_2O_5 content of the extracts was therefore determined by fusion, precipitation with molybdate solution, magnesium mixture and weighing as pyrophosphate and then calculated as distearyl lecithin. The remainder of the extractive material was estimated as neutral fats. The residue remaining after extraction was examined for Ca, Mg and P_2O_5 by the ordinary analytical methods. In making comparisons with other work, however, we must emphasize the fact that the residue of the entire aorta was used and not merely isolated calcified areas.

RESULTS: I. Calcium soaps. — As far as we were able to determine no calcium soaps, or only mere traces, were to be found in normal or pathological aortas. This was definitely shown in the following manner. Extracts of the aortas which were completely soluble in absolute alcohol gave no reaction for calcium. Extracts, with supposed calcium content, dissolved in chloroform without leaving a trace of residue. As mentioned before, the soap — calcium stearate — is insoluble in chloroform.

II. Fatty acids and lecithin. — These will be considered together.

NORMAL AORTAS.

	Dried Substance.		Fatty Extractive.		
	Per Cent CaO.	Per Cent Fatty Extractive.	Per Cent Fatty Acid.	Per Cent Lecithin.	Per Cent Neutral Fat.
1 ..	1.4	20.3	6.1	18.3	75.6
2 ..	.3	11.9	5.73	16.3	77.97
3 ..	....	....	6.18	15.9	77.92

The surprisingly large percentage of lecithin is directly in line with the results obtained by Dunham[5] in his study of the normal kidney. He found that twenty-seven to thirty-seven per cent of the fatty extract was present in the form of lecithin.

Considering now the degree of calcification in the diseased aorta (the difference in calcification can be determined definitely by difference in calcium content of the residue), a striking relationship of the fatty acids, and especially of lecithin, to the grade of calcification is noticed. This is shown in the following table, in which the analyses are arranged according to the calcium content; the last two being those in which no calcification was evident macroscopically:

	Dried Substance.		Fatty Extractive.		
	Per Cent CaO.	Per Cent Fatty Extractive.	Per Cent Fatty Acid.	Per Cent Lecithin.	Per Cent Neutral Fat.
1 ..	11.9	22.5	9.6	16.6	73.8
2 ..	7.5	30.0	13.9	19.06	67.04
3 ..	7.2	22.4	19.09	24.2	56.7
4 ..	3.8	28.3	21.9	20.9	57.2
5 ..	1.7	8.1	20.2	37.9	41.9
6 ..	1.4	9.4	25.1	52.2	22.7

It is seen that the fatty acids and lecithin are present in greatest amount in the atheromatous aortas without gross evidence of calcification and that as the calcium content increases they rapidly diminish in amount. The remarkable increase in the amount of lecithin and fatty acids which appears at the initial stage of calcification certainly lends evidence to the fact of a decided "fatty" transformation occurring at this time. The fatty acids and lecithins here may or may not have a common origin. The parallelism in their appearance points to the probability that they do. The fact that lecithin and fatty acids may arise from a decomposition of substances of the "protagon" type, which Dunham has shown to exist in surprisingly large amounts in the normal cell, further substantiates this idea. The second stage in the fatty transformation consists in the disappearance of the lecithin and fatty acids coincident with the increasing calcium content of the tissue. The fatty acids probably react with the alkalis furnished by the blood and pass out of the cell as soluble compounds. The lecithin undergoes decomposition, liberating its phosphate radical, which serves either in part or entirely as the conjugant of the calcium.

III. Neutral fats. — The remainder of the fatty extractive was calculated as neutral fats. The results are shown in the above table. This procedure is not absolutely correct, since necessarily numerous lipoids were present; for example, beautiful tests for cholesterin were obtained.

IV. The residue. — Unfortunately, it was impossible to examine only calcified areas for calcium, phosphates, and magnesium and at the same time to use all the material for fat analysis. Instead, the residue of the entire aorta was used. The entire P_2O_5 estimated was considered as combining with MgO and CaO to form $Mg_3 (PO_4)_2$ and $Ca_3 (PO_4)_2$. The remaining CaO was estimated as $CaCO_3$. In the aortas of lower calcium content there was relatively a small quantity of $Ca_3 (PO_4)_2$ while, as calcification proceeded, the proportion of calcium phosphate to carbonate increased. For instance, in an aorta with estimated 3.8 per cent Ca content, the relationship between the $Ca_3 (PO_4)_2$ and $CaCO_3$ was as 69.5 to 25; that with 7.5 per cent Ca content as 74.7 to 24.8; and that with 11.9 per cent Ca content as 79.9 to 19.9.

As the calcification proceeded, however, the magnesium content decreased, being present in the more calcified areas as 0.1 per cent and 0.32 per cent.

Comparing our results with those of Klotz and of Wells,

1. They differ from those of Klotz, in that by analytical methods we were unable to find the calcium soaps which he claims to have demonstrated by micro-chemical examination, but are in accord with Wells' results in this regard.

2. The inorganic analyses of the residues differ, however, from those of Wells. Possibly this is due to the fact that the entire aorta was used. Such material necessarily included both calcified and normal areas, thus diminishing the absolute percentage of calcification. Again Wells examined a different kind of tissue (lymph node) and it is not impossible that the somewhat higher P_2O_5 content obtained by him is due to the presence of the phosphorus containing proteids or their decomposition products, which are known to be present in such tissue in relatively large amounts. The $Ca_3 (PO_4)_2$ percentage was lower and the percentage of $Mg_3 (PO_4)_2$ much less. The figures do not

show the very close relationship to normal ossification which is so evident in his results.

CONCLUSIONS. — 1. The absence of calcium soaps in the extracts of all aortas examined leads us to the conclusion that in pathological calcification of the aorta at least, the formation of calcium soap is not an intermediate process.

2. The analyses of the residues apparently point to the fact, insisted upon by Wells, that in pathological calcification the inorganic salts are deposited in approximately the same proportion as in normal ossification. This does not necessarily signify, however, that the initial processes from which pathological calcification and normal ossification result are identical.

3. The remarkably high percentage of lecithin in the initial stage of calcification and the fact that with increasing calcium content there occurs a coincident diminution in the percentage of lecithin has led us to suspect that the phosphate radical may be supplied from a decomposition of lecithin.

[I wish here to express my indebtedness to Dr. Holmes C. Jackson, Director of the Laboratories of Physiological Chemistry and Experimental Physiology of the Albany Medical College, without whose instruction and supervision this investigation would have been impossible.]

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3. The term is meant to include all kinds of lecithins or lecithans.

4. Dunham, E. K. Proceedings of the Soc. for Exp. Biol. and Med., 1904, i, 39.

5. Loc. cit., p. 40.

THE DISTRIBUTION OF TREPONEMA PALLIDUM, SCHAUDINN (SPIROCHÆTA PALLIDA), IN THE TISSUES IN CONGENITAL SYPHILIS.*

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Since the appearance, a little over a year ago, of the communication by Schaudinn and Hoffmann[1] announcing the finding of Spirochæta pallida in the primary and secondary lesions of syphilis, the work has been confirmed by a long list of observers. The results of these observations are, in brief, that in smear preparations of the lesions of primary, secondary, and congenital syphilis one finds a characteristic spiral organism four to twenty microns long, to which Schaudinn and Hoffmann originally gave the name Spirochæta pallida. The constant occurrence of this organism in syphilis, its absence in other conditions, and its characteristic morphology, made it seem probable that the organism had some etiological relation to lues.

For its further proof, however, the question of etiological relation needed something more than the demonstration of Spirochæta pallida on smear preparations. It seemed necessary that the organism be stained in the tissues in order to determine whether it had any relation to the lesions of syphilis. Work was begun along this line, the greatest variety of stains and mordants being tried, all without success. True, Herxheimer,[2] so long ago as July, 1905, claimed to have found two individuals in a section of a primary lesion stained with nile blue, but he presented no photomicrographs, and his observations are recorded in such an indefinite fashion as to make it seem most improbable that he really had Spirochæta pallida before him. Furthermore, these observations could not be confirmed by himself, or by others with the use of nile blue. The various histological stains having

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proved negative, silver nitrate impregnation of sections suggested itself. In the meantime, there appeared the article of Bertarelli and Volpino,[3] who reported positive results after the use of a slightly modified van Ermengem method applied to the sections upon slides. We had already tried this method, and while its use is of some value in positive cases, it is extremely unsatisfactory in negative ones. Particularly after the use of reducing solutions containing pyrogallic acid, the amount of silver deposit upon the sections is very great, and this deposit usually takes the form of fine irregular spirals which prove most confusing.

In October, 1905, Levaditi[4] announced the result of a method devised by him. This procedure consists, in brief, of the silver nitrate impregnation, in bulk, of formalin hardened tissue, followed by reduction by means of pyrogallic acid. The tissue is then embedded in paraffin, the sections cut and stained with Giemsa's stain, or toluidin blue. This is the method that we have used in our work. It gives most satisfactory results and beautiful preparations. Levaditi and Manouélian's[5] later and more rapid process, in which pyridene is added to both the impregnating and the reducing solutions, seems to be better for fresh tissues removed during life, but less satisfactory for autopsy material. In our experience, counter staining with toluidin blue gives better preparations than does Giemsa's stain. By this method the nuclei are stained green. The cytoplasm and intercellular substances take varying shades of brown. The spirochætæ are colored a uniform deep black. They appear considerably thicker than when stained with Giemsa's stain. They are easily seen with a four ocular and a one-twelfth-inch immersion objective, and, after the eye has become accustomed to them, may even be found with a high dry objective. In the silver impregnated tissues the organisms retain their characteristic short, close spirals and cannot be mistaken for normal or pathological tissue constituents. In certain cases the spirals are less close than normal and more irregular, while the thickness of the organism is considerably increased. This point will be referred to later.

The two cases of hereditary syphilis included in this report are interesting because they differ clinically and histologically, as well as in the distribution of the spirochætæ.

Case 1, Autopsy 454.—A white female child dead on the fourth day after birth. The mother had had a profuse vaginal discharge for some two months before her confinement. A short while before the discharge made its appearance, her voice became hoarse, but she denies any skin eruption, sore throat, or loss of hair.

Upon her admission to hospital, there was a profuse vaginal discharge which contained numerous gonococci. On each labium majus and posterior to the fourchette, there was a superficial ulcer with reddened base, not indurated. The inguinal and other glands were not enlarged. There was no skin eruption.

Smears from the ulcerations showed an occasional example of Spirochæta refringens, none of Spirochæta pallida. Since the ulcers healed rapidly under simple cleansing and dressing, they do not appear to have been specific. The woman at the time of the birth of the child seems to have been free from all syphilitic manifestations. About three weeks after her confinement, she developed a typical macular rash. Delivery occurred during the seventh month.

At autopsy, which occurred six hours after death, the child was found to be forty-two centimeters long, emaciated, and deeply jaundiced. There were no external indications of lues. The greater part of each lung was consolidated, in the stage of red hepatization. The anatomical diagnosis was acute lobar pneumonia, cloudy degeneration of the liver and kidneys. There were thus no macroscopic lesions of syphilis present in the internal organs. The epiphyses were normal.

Smears were made from various organs and stained with Giemsa. Those from the lung showed numerous short, thick, single and diplo-bacilli, capsulated, apparently of the type of Bacillus mucosus capsulatus, but no spirochætæ. The liver showed a few bacilli of the same kind as those found in the lung. In the liver smears, Spirochæta pallida occurred in great numbers, often arranged in groups of from ten to twenty in a single field. The spleen showed a very few spirochætæ. Smears of all the other organs were negative.

Microscopically, the lung shows acute lobar pneumonia. The alveoli are gorged with red corpuscles, desquamated epithelium, and polymorphonuclear leucocytes, the latter being present in small numbers. The capillaries of the septa are distended. The connective tissue of the septa shows no change. There is a marked hemorrhagic catarrhal bronchitis. The peri-bronchial lymph spaces are dilated. The adventitia of the larger branches of the pulmonary artery appears slightly thickened, the thickening being due to a distention of the perivascular lymphatics and capillaries. In Levaditi preparations of the consolidated portions of the lung, one is struck by the numbers of spirochætæ present. In the bronchial epithelium a hundred or more organisms can easily be counted within a single immersion field (Fig. 1, Plate XXV.). Here the most common arrangement is with the long axis parallel to the long axis of the epithelial cells, giving the appearance of the active passage of the organisms through the epithelial cells. A few spirochætæ are seen in the bronchial exudate. In the sub-epithelial tissue of the bronchi, spirochætæ are as numerous as in the epithelium itself. Here they are situated between and within the connective tissue cells. The characteristic arrangement, however, is about the lymph spaces and blood capillaries. In the bronchial cartilage a few organisms occur, apparently in the matrix, and within the cells themselves (Fig. 2, Plate XXV.). In the larger blood vessels the spirochætæ occur in some numbers in the outer portion of the adventitia and about the lymphatics. They are less numerous in the inner layer of the adventitia, again becoming fairly numerous in the inner zone of the media. Here the arrangement is in part parallel with the fibers, but for the greater part at an angle to the latter, giving the picture of an active migration into the media. In the lung capillaries whose endothelium is swollen, an occasional organism is seen within the lumen and within the endothelial cells. In the alveolar septa the arrangement is peri-vascular and within the connective tissue cells of the septa. A few spirochætæ occur free in the exudate within the alveoli. Degenerated forms are seen within the desquamated alveolar epithelial cells.

The adrenal shows little histological change except congestion and swelling of its cells. This swelling is most marked in the cells of the medulla. In many of the medullary cells the nuclei stain faintly. The number of spirochætæ is astonishing (Fig. 3, Plate XXV.). They occur in equal numbers in the cortex and in the medulla, lying within the lumina of the smallest capillaries, between the parenchyma cells, and definitely within these cells. While the connective tissue of the capsule of the gland is free of organisms, they are fairly numerous in the adventitia and media of the larger blood vessels of the capsule.

The spleen, microscopically, is congested and shows a diffuse interstitial lienitis. The Malpighian bodies are very small and the lymphoid tissue in general throughout the organ is relatively small in amount. Spirochætæ are scattered about in the spleen pulp; they are present in the connective tissue trabeculæ, and are most numerous in and about the arterioles of the Malpighian bodies (Fig. 4, Plate XXV.). In the pulp the organisms are most numerous where the proliferation of the pulp stroma cells is greatest.

In the liver are many small islands of small round cell infiltration. These areas are scattered throughout the parenchyma, and while they do also occur in the connective tissue about the interlobular vessels, the process is much more widespread in the glandular tissue. The capillaries are somewhat congested. The liver cells are granular, and to some extent fatty. There is some proliferation of the interlobular connective tissue. The infiltration and new formation in this tissue do not appear to have any close relation to any of the larger vessels. Mononuclear and polymorphonuclear eosinophiles are present in the proliferating zone. Spirochætæ are not so numerous in the liver as one would suppose from the smear preparations. Their chief localization is in the proliferating interlobular tissue, in the areas of round cell infiltration, but more particularly within the more degenerated liver cells immediately about the areas of infiltration.

The heart muscle shows some fatty degeneration. Spirochætæ are fairly numerous, occurring chiefly within the muscle cells and, to some extent, about the blood vessels (Fig. 5, Plate XXV.).

The glomeruli of the kidney are congested. The epithelium of the convoluted tubules is swollen and granular. The spirochætæ are localized in greatest numbers in the interstitial tissue. They occur also within, between, and beneath the epithelial cells of the tubules.

In the thymus, which shows nothing abnormal except a decrease in the number of large lymphoid cells, patient searching is rewarded with the finding of an occasional organism in the interlobular connective tissue, or within a capillary.

In the pancreas the search is almost as wearisome, but a few spirochætæ are seen in the interlobular tissue, and two within the lumen of a large vein. The gland cells are somewhat swollen. Blood vessels, connective tissue, and islands of Langerhans show no change.

We may summarize this case as follows:

A child, born alive, of a mother who did not develop secondary manifestations until after her confinement, and who most probably contracted her infection during her pregnancy.

The child shows only slight, but undoubted, lesions of syphilis, while the entire body is flooded with spirochætæ. This is a condition comparable to a bacteriemia, in which death occurs before there is time for the development of marked pathological lesions.

The fact that the liver shows the most advanced changes would indicate a transference of the infection from the placenta by means of the umbilical circulation. The further spread seems to be by the blood capillaries, and, possibly to a greater extent, by the perivascular lymphatics.

Case 2, Autopsy 449. — A white male infant, born dead three weeks prematurely. The mother was a foreigner, and her history was not obtainable. Upon admission to St. Ann's her body was covered with the copper-colored spots so characteristic of an old syphilitic rash. The hair and the eyebrows were thin. Soft, broad condylomata were present about the gentalia and the anus. The fetal heart sounds could not be heard at the beginning of labor.

At autopsy, which occurred twenty hours after delivery, the child was found to be well nourished, and forty-four centimeters long. The weight was nineteen hundred and forty grams. The skin was peeled off in many places, and the subcutaneous tissue was markedly edematous. There were no bullæ present and no skin eruption. The anatomical diagnosis was fetal syphilis, based upon the following findings: Interstitial lienitis, syphilitic osteochondritis, gumma of the lung.

The placenta weighed five hundred and sixty grams; it showed nothing abnormal macroscopically except several white infarcts. The umbilical vein was thickened, surrounded by a narrow, grayish, opaque zone.

Smears from the various fetal organs, from the placenta, and from the tissue and blood of the cord, stained with Giemsa's stain, were negative. Examination of a great number of preparations failed to disclose a single organism.

Histological examination: The lung is airless, the alveolar walls thickened. Scattered about in the alveolar septa are small areas of round cell infiltration. The walls of the blood vessels are thickened and edematous. The lung tissue is fairly well preserved. The area described macroscopically as a gumma is composed of a relatively large center of coagulation necrosis, surrounded by a thin wall of dense connective tissue. Next this layer of fibrous tissue the alveolar walls are infiltrated with round cells. Spirochætæ are fairly numerous, occurring in greatest numbers in the walls and perivascular tissue of the thickened blood vessels. They are present also in the peri-bronchial tissue, in the areas of round cell infiltration, and in the thickened alveolar septa. An occasional organism is seen within an endothelial cell of a blood vessel. In the gumma none can be seen in the necrotic tissue. They are very rare in the fibrous wall, somewhat more numerous in the surrounding thickened and infiltrated alveolar tissue.

The liver is very necrotic and granular, the nuclei staining very poorly. The organ is divided into small islands of liver cells by an edematous connective tissue stroma. The walls of the larger vessels are thickened and edematous. The walls of the smaller vessels are also thicker than normal, the lumina almost obliterated. The thickening of the walls of these smaller vessels is due in part to edema, in part to round cell infiltration. A few spirochætæ are seen in the walls of the blood vessels and an occasional irregular one in the interstitial connective tissue of the organ.

The spleen shows a marked increase of interstitial tissue. The larger pulp cells are swollen, granular, and contain blood pigment. Their nuclei are poorly stained. Small lymphoid cells are few in number and also show evidences of beginning degeneration. The trabeculæ and blood vessel walls are thickened. Spirochætæ are fairly numerous, being located in the walls of the larger blood vessels and about the arterioles of the Malpighian bodies. An occasional one is seen free in the pulp. In the latter place, they are irregular and thicker than normal. About the arterioles, where the tissue is better preserved, they retain their normal regularity and size.

The kidney is also markedly degenerated, and shows an increase in the connective tissue about the glomeruli and blood vessels, and between the tubules. Spirochætæ occur in some numbers in this proliferating connective tissue. They are fewer and less well preserved in the tubular epithelium.

In the adrenal, which is considerably increased in size, the maceration is even more advanced than in the liver. The medulla is infiltrated with blood and shows a few areas of round cells, whose nuclei still stain fairly well. Most of the cells of the medulla are entirely degenerated. This degeneration is somewhat less marked in the cortex, but even here the cells are granular and their nuclei do not stain. The entire organ is edematous, and there is some increase in the amount of stroma in the medulla. Spirochætæ are more numerous in the adrenal than in any other organ. Most of them, particularly in the more degenerated areas, are swollen, the spirals irregular and further apart than normal, and often absent over the greater part of the organism. Many of the organisms show swollen, round, knob-like ends, while others show swellings along the course of the body (Fig. 6, Plate XXV.). In some cases this polar enlargement appears to be due to a definite rolling in upon itself of the end of the spirochæta. Other examples are completely rolled upon themselves, but in these the coils are more loosely arranged. In the better preserved parts of the gland the organisms retain their normal appearance. Throughout the silver nitrate sections of the adrenal there is scattered a considerable amount of finely and coarsely granular black material.

The lower end of the femur shows a typical irregular ossification line, characteristic of syphilitic osteochondritis. Spirochætæ are very rare. They lie among the cells of the marrow.

The placenta shows little change except thickening of the walls of the vessels of the primary villi, and some increase in the density of the stroma of the villi. In the peripheral portion of the thickened vessel walls an occasional organism is seen.

The stroma of the umbilical cord is somewhat increased in density. About the vein there is a zone of cellular infiltration, a condition recently described by Thomsen.[7] In these areas the cells are degenerated, the nuclei undergoing rhexis. Among these cells a very few spirochætæ can be found.

This case may be summarized thus:

A premature, still-born infant, showing advanced internal manifestations of syphilis and beginning maceration. From these two facts, as well as from the mother's history, infection of the fetus must have occurred at conception, or early in pregnancy.

Spirochætæ occur in much fewer numbers than in the first case. This may be due to the amount of autolysis present in the tissues. It seems more plausible, however, to consider the infection a much lighter one from the beginning, one causing gradual changes in the organs and permitting a development almost to term.

The rarity of spirochætæ in the placenta agrees with the findings of Paaschen,[8] of Menetrier and Rubens-Duval,[9] of Nattan-Larrier and Brindeau,[10] and of Wallich and Levaditi,[11] who found that Spirochæta pallida is present in the placenta only when the child is born with manifest syphilitic lesions, and then only in small numbers.

The presence of spirochætæ in the living tissues of the gumma and not in the necrotic tissue is interesting. Reuter[6] reports a similar finding in Levaditi preparations of a gumma of the lung in congenital syphilis. Gummata of acquired syphilis have been uniformly negative. In my own work, smears and silver impregnation sections have been negative in three such gummata. This failure to find the spirochætæ is probably due to the small number of organisms present in such lesions. Metchnikoff and Roux have shown that gummata are infectious, but that large amounts of material are necessary for successful inoculation.

A point of interest is the irregularity of form presented by the spirochætæ in the second case (Fig. 6, Plate XXV.). Levaditi[12] has pointed out a similar condition in a macerated fetus studied by him and suggests that this may mean a beginning degeneration of the parasites. This view is a very probable one, since these irregular, swollen, and knobbed specimens occur where the tissue necrosis is most marked. Yet these organisms with enlarged ends remind one much of similar stages described by Perrin[13] in Spirochæta Balbiani and by von Prowazek[14] in Spirochæta gallinarum. The former states definitely that the condition is one of cyst formation and reproduction. The latter also asks whether the condition may not be due to a reproductive act, the result of conjugation. Forms completely rolled upon themselves have also been described by both observers in the species studied by them. That the forms pictured by Perrin are reproductive forms is denied by Mesnil,[15] who considers them stages in the involution of the parasite.

In the first case, smears from the various organs, except the liver and spleen, were negative, in spite of the great number of organisms present in the sections of almost all of the organs. In the second case all smears were negative. The negative findings in smear preparations in a suspected case cannot, therefore, be considered conclusive. In order to exclude positively the presence of Spirochæta pallida in a lesion, sections are necessary. Mucha and Scherber[16] have also pointed out this discrepancy between the findings in smears stained by Giemsa and in sections impregnated with silver.

The comparative rarity of spirochætæ in smears from the organs, which show numerous specimens in sections, must be due to the intracellular location of the parasites. In the liver, the lung, the adrenal, and the kidney there cannot be the least doubt of the presence of the spirochætæ within the cells. And in all the various tissues the connective tissue cells also harbor parasites. The liver in the first case shows a marked preference on the part of the organisms for the gland cells, a point to which Levaditi[12] has called attention. Spirochæta pallida must be considered by choice an intracellular parasite. Whether the increase of connective tissue in syphilis, as well as in other chronic inflammations, is due to a change that is primary in that tissue, or whether it is due to a compensatory proliferation whose aim is the replacement of primarily injured parenchyma cells, is a question which has received much discussion in pathological literature. In congenital syphilis the primary change seems to be an infection of parenchyma cells, leading to the injury and death of those cells. This is shown particularly well in the liver of Case I. Since the connective tissue cells do also contain spirochætæ, a primary stimulation of the connective tissue cannot be excluded. The passage of the spirochætæ through the walls of the blood vessels and their multiplication in the perivascular lymph spaces explain the vascular changes which are so marked in syphilis.

The presence of spirochætæ within the cytoplasm, particularly of the epithelial cells, cannot be considered a phenomenon of phagocytosis. The parasites stain well and have, apparently, undergone no change. The infected cells, on the other hand, show changes varying from cloudy degeneration to complete necrosis. In the bronchial epithelium of Case 1, this active invasion of the cells is well shown.

That a phagocytosis does occur Levaditi[12] has shown. In Observation 5 of his series the mononuclear macrophages within the lung alveoli contain parasites in various stages of degeneration. The lung of our Case 1 shows a similar condition. Many of the intra-alveolar cells contain spirochætæ which do not stain regularly, and some which are fragmented.

A question almost as interesting as the etiological relation to syphilis of Spirochæta pallida is that of the biological position of the organism. Does it belong to the Protozoa, or to the Bacteriaceæ? In Schaudinn and Hoffman's[1] earliest communications they classed it among the spirochætæ. The type species of this genus, proposed by Ehrenberg in 1834, is Spirochæta plicatilis. The genus can, therefore, include only such species which answer to the morphology of Spirochæta plicatilis. This organism, according to Schaudinn,[17] has a periplast wound spirally about an elongated body in the form of an undulating membrane. It has no flagella. The body shows a small number of irregular spirals which are not fixed and which may disappear and reappear from time to time. Schaudinn describes a larger central chromatin mass, which he thinks is the kinetic nucleus. Scattered about upon this in the form of chromidia are numerous small chromatin granules, which he considers the vegetative nucleus. The ends are somewhat rounded. The same observer describes a similar morphology for Spirochæta refringens, and for a spirochæta obtained from an ulcerated carcinoma. He also placed the organism of relapsing fever in the same genus. The recent work of Zettnow,[18] of Norris, Pappenheimer and Flournoy,[19] and of Novy and Knapp,[20] makes the correctness of Schaudinn's views regarding Spirochæta Obermeiri appear extremely doubtful. None of these authors could observe any differentiation within the cell body, and they could not demonstrate an undulating membrane. All agreed that multiplication occurs by transverse fission. Novy and Knapp state further that toward plasmolysing reagents Spirochæta Obermeiri reacts like the bacteria, and not like the protozoa. The latter organism cannot be retained in the genus Spirochæta. Simply because this one example of the "spirochete group" must drop out of the genus is not, as Novy and Knapp would seem to intimate, conclusive enough proof of the bacterial nature of all the rest of the organisms described as spirochætæ. Because of conflicting observations and of divergent opinions, the question of the biological position of the true spirochætæ, that is, those answering to the type species, Spirochæta plicatilis, is much confused. One organism which will serve as an example of the existing confusion is Spirochæta gallinarum. Borrel[21] describes peritrichous flagella, transverse division, and the absence of an undulating membrane. The more recent observations of von Prowazek[14] do not agree in any particular with those of Borrel. The former was able to demonstrate an undulating membrane and longitudinal division. He could not produce the plasmolysis characteristic of the bacteria, nor could he observe the flagella described by Borrel. That the latter's work "definitely relegates this organism to the group of bacteria" (Novy and Knapp, l. c., page 299) must, therefore, appear doubtful. The protozoan nature of the spirochætæ seemed to receive conclusive proof by Schaudinn's[22] work upon the blood parasites of the owl, Athene noctua. He described a complicated alternation of generations between Hemamœba Ziemanni, a trypanosome stage, and a spirochæta stage. In a later communication,[17] however, he recedes from this position and states that the forms described as Spirochæta Ziemanni are true trypanosomes. He still, however, contends for the protozoan nature of the spirochætæ. The grounds brought forward are the complicated, dual, nuclear structure, the presence of an undulating membrane, and longitudinal division. An undulating membrane is described by Schaudinn[17] for Spirochæta refringens, Spirochæta plicatilis, and the spirochæta of an ulcerated carcinoma; by Perrin[18] for

Spirochæta Balbiana; by von Prowazek,[14] for Spirochæta gallinarum; and by Keysselitz,[23] for Spirochæta anodontæ. A dual nuclear apparatus, similar to that described by Schaudinn in Spirochæta plicatilis, is observed by von Prowazek in Spirochæta buccalis, and by Keysselitz in Spirochæta anodontæ. That a distribution of the chromatin, similar to the condition found by Schaudinn in Spirochæta plicatilis, is absolute proof of the protozoan nature of the organism may be doubted. Bütschli[24] described a very similar nuclear structure for Spirillum volutans. The occurrence of longitudinal division, however, would place the spirochætæ definitely among the protozoa. Such a form of division is described by Perrin for Spirochæta Balbiana; by von Prowazek for Spirochæta gallinarum and Spirochæta buccalis, and by Keysslitz for Spirochæta anodontæ.

In the same communication in which Schaudinn[17] gives more minutely the characteristics of Spirochæta plicatilis, he states that the organism originally named by him and Hoffmann, Spirochæta pallida, has none of these characteristics. There is no undulating membrane, and there is a fine flagellum at each end. Since Spirochæta pallida is not a spirochæta, what is it? Before the appearance of this paper by Schaudinn, Vuillémin had already suggested the name Spironema, which Schaudinn[17] accepted. Stiles and Pfender[26] pointed out that this name had been preëmpted by a genus of Mollusca, and put forward the name Microspironema. However, before the appearance of their publication, Schaudinn[27] had also discovered the incorrectness of the name Spironema, and proposed the name Treponema.

If Treponema pallidum is not a spirochæta, is it a protozoon? Morphologically it seems to approach very nearly Spirillum (Ehrenberg, 1830).

According to Thesing[28] the polar flagella and the regularity of the spirals of Treponema pallidum are proof of the bacterial nature of the organism. Goldhorn,[29] because of the appearance of transverse division, comes to the same conclusion. Wechselmann and Loewenthal[30] think that the longer forms break up into two or more short organisms.

This would indicate transverse division. By means of the ultra-microscope they claim to be able to see a definite nucleus. In a later communication[31] they think that the association of spirochætæ and fusiform bacilli on smears indicates a transformation of one stage to the other, a condition which, according to Tunnicliff,[32] occurs in the fusiform bacillus and the spirochæta of Vincent's angina. In their later paper Wechselmann and Loewenthal state that the spirochæta divides into a number of short rods, each of which develops a definite nucleus. These rods grow and become bent, while the nucleus divides to form a chromidial network.

Schütz's attempt to establish a life cycle goes even further. He claims to find both Treponema pallidum, and Cytoryctes luis, Siegel, in syphilitic lesions, and thinks they may be stages of the same organism. Because of the frequency with which Spirochæta refringens and Treponema pallidum are associated, he suggests that the former may represent a macrogamete stage, the latter a microgamete. This view can have little value, because of the constant occurrence of Treponema pallidum in syphilis and only in syphilis, and the presence of Spirochæta refringens in the greatest variety of non-specific lesions.

Herxheimer[2] described at the end of the organism a body which he compared to a centrosome. Further, he described scattered, fine, chromatin granules which he considered analogous to the kinetic nucleus or blepharoplast of the trypanosomes, and larger chromatin granules which were supposed to represent the vegetative nucleus. He also described granules lying at a slight distance from the body of the parasite, supposedly part of the periplast or undulating membrane. In a later communication, Herxheimer and Opificius[34] modify the former statement to some extent, in that they think that the granules near the body have nothing to do with the organism. In sections they describe y-shaped organisms, which they think indicate longitudinal division, and rosette forms due to agglomeration. Krzysztalowicz and Siedlecki[35] describe a cycle which would place Treponema pallidum among the trypanosomes. They describe forms which they style "formes spirochetiennes." For these they describe a clear central vacuole, which represents the nucleus. Division is longitudinal, and occurs in those organisms which have increased in thickness. In the "formes trypanosomiques" there is a central nucleus, closely approximated to which is a small chromatin granule (the blepharoplast). From the latter a fine filament runs to the extremity of the body; this represents a rudimentary undulating membrane. These forms also divide longitudinally. Stages representing conjugation are also described. A very serious objection to their work is the fact that their smears were derived, in part, from ulcerated and secondarily infected lesions.

The chief objection to the work of these various observers is their very evident desire to give to Treponema pallidum a life cycle analogous to that of the organism to which the individual observer thinks Treponema is most nearly related.

So much of our knowledge concerning Treponema as is beyond dispute may be summarized as follows: A spiral organism four to twenty microns long, four-tenths micron broad, sharply pointed at each end. The spirals are close and regular. The length of the individual coils averages one micron. During periods of cessation of locomotion the coils may disappear and reappear. There is no undulating membrane, and the body shows no nuclear differentiation. At each end there is a fine flagellum.

According to Schaudinn the things which speak in favor of the protozoan nature of Treponema pallidum are the possession of a single polar flagellum at each end instead of a group of flagella; the large number of spirals and their flexibility, as opposed to the small number of spirals and their inflexibility in the spirilla; and finally, the probability of longitudinal division.

If Treponema pallidum is a protozoon, it probably belongs to the class Flagellata, and satisfactory evidence of the longitudinal division of the parasite would be positive proof of its protozoan nature. Schaudinn[17] has described organisms with two flagella at one pole. He considers this the beginning of a longitudinal division. Goldhorn[29] believes that the division is transverse. His figures (4 and 9 on Plate XXII.) cannot be satisfactorily explained as examples of the simple mechanical intertwining of two individuals. The liver smears from the first case here reported show closely intertwined pairs which look like stages of a division by longitudinal fission. If transverse division does occur, one would expect to see undoubted evidences of it in the tissues. In silver impregnated specimens the organisms can be seen much more readily than on smears, and one ought to be able to find clear-cut examples of transverse fission, if such a process does occur. In none of my sections have I been able to find anything which could be thus interpreted. On the contrary, one does often enough find examples in which two organisms appear to be closely united for a distance of one half or one-third the length of the body. I have been unable to detect any evidence of intertwining in such specimens with the use of Zeiss 12 compensation ocular and 1/18 objective. If such pictures are due to the union of two organisms the united portion ought to be somewhat thicker than the combined diameters of the two, because of the deposition of silver between them. Such is not the case. The united portion is somewhat thicker than a single organism, but is not equal in thickness to the sum of the diameters of two.

CONCLUSIONS.

The two cases here reported confirm the findings of Levaditi,[12] of Buschke and Fischer,[36] of Mucha and Scherber,[16] of Herxheimer and Opificius,[34] of Gierke,[37] and of Paaschen.[38]

In sections the organisms have the same characteristic morphology that they have in smear preparations.

Treponema pallidum bears a definite relation to the lesions of hereditary syphilis.

It is, to a marked degree, an intracellular parasite, glandular epithelium being particularly vulnerable.

The connective tissue new-formation, so characteristic of syphilis, is due in part to this destruction of parenchyma cells, in part to a stimulation of the connective tissue.

In those cases of congenital syphilis in which the mother is diseased, infection occurs by way of the placental circulation.

Multiplication of Treponema pallidum occurs chiefly in the perivascular lymphatics and within the tissues themselves; not within the larger blood vessels.

The peri-arteritis, which occurs in syphilis, is due to this perivascular localization of the organism.

The protozoan nature of the organism has not been proven. It does, however, appear probable.

[My thanks are due to Dr. R. G. Perkins for the preparation of microphotographs; and to Dr. H. M. Tarr, the resident obstetrician at St. Ann's Maternity Hospital, for material. It is a pleasure to express my gratitude to Prof. W. T. Howard, Jr., for encouragement and for helpful suggestions.]

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DESCRIPTION OF PLATE XXV.

(*All microphotographs were made with the aid of a 1/12 Leitz objective and No. 4 ocular. Bellows length, 55.0 cm.*)

FIG. 1, Case 1. Lung, epithelium of bronchus.

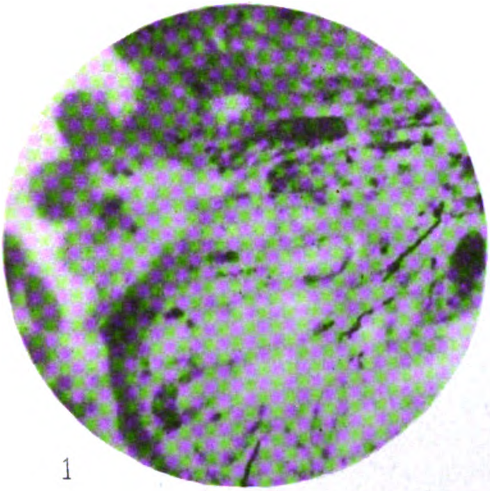
FIG. 2, Case 1. Lung, bronchial cartilage.

FIG. 3, Case 1. Medulla of adrenal.

FIG. 4, Case 1. Spleen, arteriole of Malpighian body.

FIG. 5, Case 1. Heart muscle.

FIG. 6, Case 2. Cortex of adrenal showing necrosis. Treponema with swollen ends.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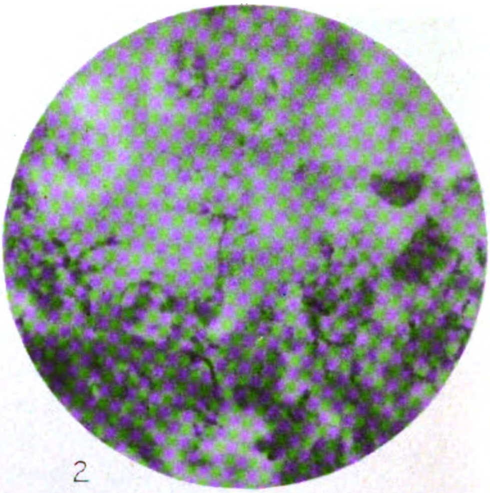
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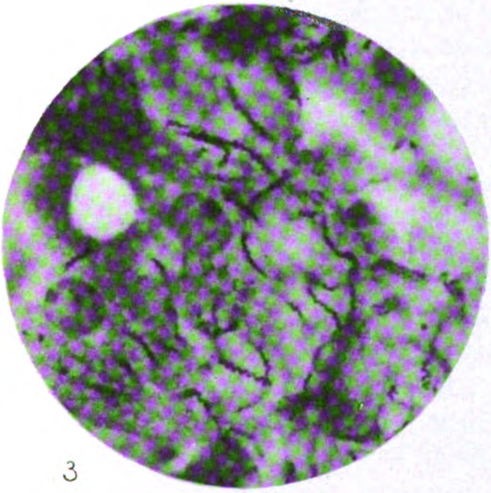
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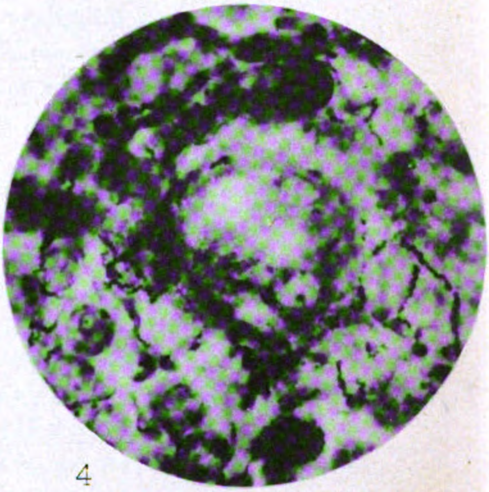
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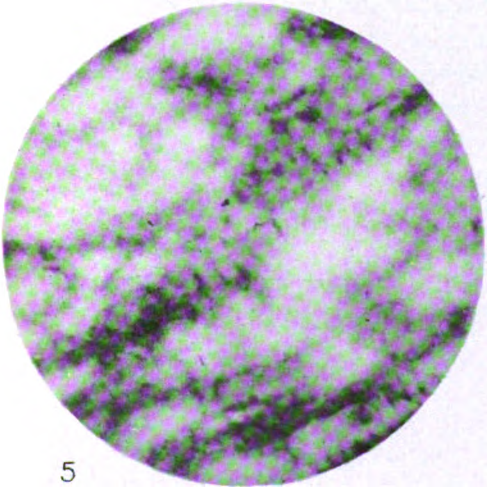
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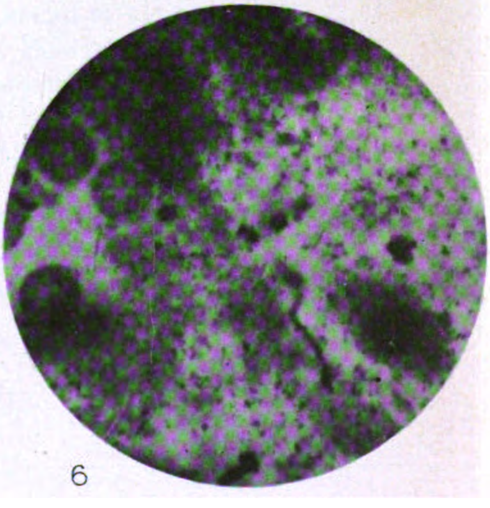
6

Schultz.

Treponema pallidum, Schaudinn.

THE EFFECT OF AMYL NITRITE INHALATIONS UPON THE BLOOD PRESSURES IN MAN.*

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Numerous studies upon animals have shown that small doses of amyl nitrate affect the circulatory apparatus in two main ways. In the first place, the heart action is quickened, owing to a diminution or loss of that constant inhibitory influence which is exerted by the vagus center upon this organ. Freed of this controlling influence, the heart tends to take up the same rate as it would were both vagi cut.[1] In the second place, there is a rapid and marked fall in blood pressure, owing principally to a dilatation of the peripheral blood vessels.[2] Winkler[3] has attempted to estimate the effect of amyl nitrite upon the work of the heart by simultaneously measuring the pressures in the aorta and in the left auricle. He found that the fall of pressure in the aorta was not associated with a proportionate fall in the auricular pressure and that it may even be associated with a rise of pressure in the auricle. From these data he argues that the heart does less work when under the influence of amyl nitrite. This conclusion seems to me to be justified in no way, for the amyl nitrate by dilating the arteries might easily cause a more rapid flow of blood and consequently raise the venous and auricular pressures. The work imposed upon the heart in pumping this excessive amount of blood against a lowered pressure might be greater than when less blood was pumped against the normal pressure. Furthermore, Winkler worked with comparatively large doses of amyl nitrite and for this reason also his results should not be transferred too hastily to man. Large doses of amyl nitrite, greatly in excess of those used therapeutically, directly weaken the hearts of animals.[4]

It has been generally assumed that the fall of pressure

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seen in animals likewise occurs in man; yet the actual observations upon this point, coming to my notice, are more or less contradictory. Schweinberg[5] states that minimal doses of amyl nitrate increase the blood pressure, whereas larger doses diminish it. Maximowitsch and Rieder[6] working with a v. Basch instrument obtained very considerable increases of blood pressure after amyl nitrite inhalations; *e.g.*, a rise from eighty to one hundred and forty millimeters of mercury.

As amyl nitrate is widely used among physicians to lower the blood pressure, the question as to its action is rather important. The present research was undertaken at the suggestion of Dr. J. O. Hirschfelder with a view to adding to our knowledge of the action of amyl nitrite inhalations upon the circulatory apparatus in man.

In determining the blood pressures I have used the Erlanger[7] apparatus with a ten-centimeter cuff.[8] This instrument possesses the advantage over other blood pressure instruments of yielding graphic records, which latter reduce the personal equation of the observer to a minimum. Furthermore, the principle involved differs somewhat from that of the more widely used Riva-Rocci method. The Erlanger instrument indicates the point at which the blood first begins to pass beneath the pressure cuff, whereas the Riva-Rocci method determines the point at which sufficient blood passes beneath the cuff to cause a palpable pulse at the wrist. Obviously less pressure is exerted upon the brachial artery in the latter instance than in the former, the actual difference being five to fifteen millimeters of mercury. If the artery beyond the cuff be dilated, then this difference in results between the two methods will be relatively increased, for relatively more blood must enter a wide artery at each beat before a pulse is produced at the wrist. This fact introduces a considerable source of error into the study of the action of amyl nitrite and similar drugs by the Riva-Rocci method. I have found by comparing the two methods in a few cases that the Riva-Rocci method not only gives low readings under normal circumstances, but that it gives relatively much lower readings after the use of vaso-dilators.

Although the vaso-dilator action of amyl nitrite has been demonstrated conclusively on animals, and with less certainty on man, nevertheless it may be of interest to describe an experiment which not only supports the view that amyl nitrite dilates the vessels, but also demonstrates a possible source of error in plethysmographic experiments. It was found in a number of individuals that the forearm and hand shrink more or less in the plethysmograph during the early stages of an amyl nitrite inhalation (Fig. 1); *i.e.*, blood leaves the hand for other parts of the body. During these determinations the arm was held above the level of the rest of the body.[9] Since, however, the results thus obtained were not easily explained, I decided to reverse the position of the hand, allowing it to fall below the level of the remainder of the body. It was then found that an amyl nitrite inhalation caused the dependent hand and forearm to swell. Plainly the action of the amyl nitrite in these experiments was to relax the blood vessels in the hands and arms as well as in some other portions of the body, which would allow relatively more blood than normal to sink to dependent parts.

My conclusions as to the effect of amyl nitrite upon the systolic and diastolic pressures in man are drawn from thirty-three observations upon sixteen different individuals. The latter were young men for the most part, either absolutely healthy or suffering from some relatively slight complaint. Such individuals appear to react more typically to amyl nitrite than do older men. From three to five minims of the drug were inhaled, the duration of the inhalation being one to two minutes. The inhalation nearly always produced a flushed face and sensations of fulness and throbbing in the head.

The tracings on the Erlanger drum were made as rapidly as possible one after another. The drum rotated continuously and the time was indicated by a second marker (Fig. 2). From the data thus obtained, curves of systolic and diastolic pressures were constructed. Owing to the rapid alterations of pressure these curves were drawn from comparatively few points; yet the generally uniform character of the curves is sufficient evidence of their approximate correctness.

In the typical curve (Figs. 3 and 4) the systolic pressure falls rapidly after the beginning of the inhalation and reaches a minimum in from thirty to sixty seconds. The extent of this primary fall varied up to forty millimeters of mercury, the average being thirteen millimeters. In three observations no primary fall occurred. Directly following this fall the systolic pressure rises rapidly, and usually passes considerably above the original pressure. This secondary elevation of pressure varies from 0 to twenty-eight millimeters of mercury, the average being fifteen millimeters. The maximum of this secondary rise usually occurs from one to three minutes after the beginning of the inhalation. In a few cases the maximum was not reached until four to five minutes. From this maximum the systolic pressure gradually falls again and usually reaches the normal level five or six minutes after the beginning of the inhalation. Sometimes the subsequent pressure is slightly lower than before the use of the amyl nitrite.

In typical cases the diastolic pressure falls with the primary fall in systolic pressure, but to a relatively less extent; rarely exceeding ten or fifteen millimeters of mercury. From this minimum the diastolic pressure then gradually rises to the normal, reaching this two or three minutes after the inhalation began. More rarely there is a secondary elevation in the diastolic curve corresponding to the secondary rise in the systolic curve; but the secondary rise in the diastolic when present is relatively much less and the normal level is soon reached.

The pulse pressure, or difference between the systolic and diastolic pressures, is difficult to estimate under these rapidly changing conditions; for it is manifestly impossible with a single instrument to determine the systolic and diastolic pressures at the same moment. I have estimated the pulse pressure by verticals between the systolic and diastolic curves. This method, though only approximate, has yielded fairly concordant results. The pulse pressure usually falls slightly with the primary fall in systolic pressure; for, as we have seen, the systolic fall exceeds the diastolic fall in most cases. With the secondary rise in systolic pressure the pulse pressure likewise rises; but here very rapidly and to a considerable extent, for at this time the diastolic pressure continues below the original level. This maximum of pulse pressure in the second or third minute is in some instances almost twice the normal pulse pressure for the individual. From this maximum the pulse pressure again falls gradually and finally reaches the normal five or six minutes after the inhalation.

It is ordinarily impossible to draw any definite conclusion from the extent of the movements of the recording lever in the Erlanger instrument because each adjustment of the instrument affects the movements of the lever. In a general way, however, it was noticed that the extent of the excursions of the lever in different individuals varied directly with the pulse pressure. After amyl nitrate inhalations, the excursions of the lever vary fairly uniformly (Fig. 2). They usually diminish with the lessened pulse pressure of the first minute and increase quite markedly with the coincident increase of pulse pressure during the second and third minutes. These changes are probably not due to the changes in pulse pressure alone, but are influenced by the relaxed state of the arteries. Wider arteries when subjected to a pressure approximately diastolic would make wider excursions even though the variations in blood pressure within them remained the same.

The pulse rate after amyl nitrite inhalations increases rapidly in typical cases during the first half minute, reaching a maximum at about one minute (Figs. 4–6). This maximum may be nearly twice the original rate. After the maximum the pulse rate falls rapidly, becomes normal during the third minute, and remains normal after this. In two old men who had inhaled amyl nitrite this variation in the pulse rate was much less marked than usual, and in one of these (Fig. 7) the rate increased only from fifty-four to sixty per minute, although the systolic pressure had fallen thirty-five millimeters of mercury. Such observations are in accord with the explanation that the increased heart rate is due to a diminution or loss of the vagus tone; for we know[9] that the vagus control in old people is relatively slight and that consequently a loss of that control should not increase the pulse rate so markedly as it does in the young.

In what way does amyl nitrite affect the circulation and the amount of blood propelled by the heart? In attempting to answer this question we immediately encounter the more fundamental problem as to the meaning of the pulse pressure. Unfortunately no complete experimental observations have been made on the factors that influence pulse pressure. From what is known, however, one may conceive that this is influenced in at least three ways: (*a*) by the quantity of blood expelled at each systole of the left ventricle, the greater the quantity the greater the difference between the systolic and diastolic pressures; (*b*) by the rapidity with which this blood is expelled, the more rapid the expulsion the greater the pulse pressure; (*c*) by the size and elastic resistance of the arteries, the smaller and more rigid the arteries the greater will be the effect of the expulsion of a given amount of blood in a given time. To my mind most other factors may be resolved into these components.

The cause of the varying pressures within the arteries is the intermittent character of the expulsion of blood from the heart, and these variations become less and less as we proceed toward the periphery, until finally in the capillaries we find a nearly uniform pressure and a nearly constant flow of blood. We may regard the arteries, therefore, as an elastic system which transforms an intermittent pressure into a constant one. In a somewhat similar, purely hypothetical system I have been able to work out a mathematical formula for the determination of pulse pressure. Assume that the intermittent outflow from a pump is converted into a continuous flow by an elastic bulb.

Let v = the volume of fluid driven out at each stroke of the pump.
t = the time in seconds elapsing between the beginning of one stroke of the pump and the beginning of the next.
t' = the time in seconds taken up in emptying the pump (time of systole).
Then v/t is the rate of flow per second beyond the bulb.
$t'.v/t$ is the amount of fluid that leaves the bag during the time of systole.
If B be the volume of the elastic bag at the lowest pressure (diastolic volume),
Then the volume of the bag at the highest pressure (systolic volume) will be $B + v - t'.v/t$: — *i.e.*, it is equal to the diastolic volume plus the amount of fluid entering the bag during the stroke of the pump and minus the volume leaving it during the same time (during the time of systole).
Now if the elasticity of the bag is such that equal increments of pressure within the ranges under consideration cause equal increments of volume (according to Hürthle,[10] this supposition is true for the dog's aorta between the pressures of sixty and one hundred and eighty millimeters of mercury),
Then diastolic pressure $= k.B$;
systolic pressure $= k(B + v - t'.v/t)$, k being a constant, depending on the elasticity of the bag,
pulse pressure or difference between the systolic and diastolic pressures $kv - k.t'.v/t = k.v.(1 - t'/t)$;
That is, the pulse pressure is equal to the volume of fluid expelled at each stroke of the pump, multiplied by a constant, depending on the elasticity of the bag, multiplied by the factor

$$1 - \frac{\text{time of systole}}{\text{time of the total beat.}}$$

For a number of reasons this formula cannot be applied directly to the arterial system in man. The constant flow from the distal end of the bulb is purely hypothetical, and could not occur if the pressure within the bag varied greatly. Furthermore, the arteries constitute a system of elongated elastic tubes, and the changes of pressure pass over them in waves, so that the pressure in different parts of the arterial system may vary in different directions at one and the same time. However, in spite of these objections, the general resemblance of our hypothetical system to the circulatory apparatus in man is evident, and the formula obtained for the one certainly applies to some extent to the other.

Returning to the question of the action of amyl nitrite inhalations, the main factor influencing the pulse pressure in a given individual is probably the volume of blood delivered at each systole of the heart. On this assumption the slight fall in pulse pressure, usually seen during the first minute after the inhalation, would mean that slightly less blood was being expelled from the heart at each systole. At this time, however, the heart rate is already on the increase, and this increase is so much greater than the usual fall of pulse pressure that the total amount of blood expelled during this period would exceed the normal. In the second and third minutes when the heart rate is returning to the normal the pulse pressure is much increased, even to twice the normal. At this time, therefore, the output at each systole is greatly augmented.

If this line of reasoning were correct, then one would expect that the increased movements of the left ventricle in favorable cases would be visible in an X-ray screen examination. The increased heart rate during the first minute was plainly visible in three patients examined. In one there was possibly a slight increase in the extent of the movements during this rapid action but no noticeable increase in the extent of the movements during the subsequent slowing. No determinations of the pulse pressure had been made on this individual. The two others showed the typical changes of pulse pressure and the secondary rise was particularly well marked in the last of these. In both, the extent of the movements of the left ventricle were increased during the period of high pulse pressure. On one patient this increase was observed by three observers simultaneously, and it was confirmed by repeating the inhalation. Dr. Lehmann's note on the last patient is as follows: "Heart shadow normal, arch of the aorta bulging slightly if at all. One-half minute after the inhalation the action of the heart became decidedly more rapid. As the action of the heart slowed down, the excursions of the left ventricle became wider by about one-half centimeter, but they soon returned to the normal. No change in the shadow of the right side of the heart could be made out." An increase in the extent of the movements of the left ventricle would of course indicate an increase in the volume of blood expelled at each systole. We see, therefore, that the X-ray screen examination confirms the inference drawn from the pulse pressure as to the increase in the systolic output of the heart during the second stage of amyl nitrite action.

Amyl nitrite, therefore, increases the total output of blood from the heart both during the primary fall of systolic pressure and during the secondary rise. During the former, the increased output is due to the more rapid heart action; during the latter it is due mainly to a greater amount of blood expelled at each systole. It would appear, therefore, that although amyl nitrite dilates the peripheral blood vessels it does not necessarily reduce the blood pressure to a like degree. The latter is avoided by the increased output of blood from the heart. The typical action of amyl nitrite is not that usually ascribed to it; viz., dilatation of the peripheral arteries and fall of blood pressure. It does, indeed, dilate the blood vessels, but it also causes the heart to pump more blood, and the result is a more rapid flow of blood through the body.

When the effect of a drug is so complex as is that of amyl nitrite upon the circulatory apparatus, it is but natural to expect that some variations from the typical action should occur; and such were indeed observed. I have already mentioned that the pulse rate in old persons, whose hearts are comparatively free from vagus control, does not become so accelerated after an amyl nitrite inhalation as it does in the young (see Fig. 7). The primary fall in systolic pressure in two instances was extreme (forty and thirty millimeters of mercury) and it was associated with a correspondingly great fall of pulse pressure (Fig. 5). At the time of this fall the pulse was hardly palpable and the patients seemed prostrated. Such a marked fall of pulse pressure would indicate that each systole of the heart expelled much less blood than usual, so much less, indeed, that in spite of the increased rate the circulation was probably materially slowed. One cannot but think that in these individuals the amyl nitrite temporarily weakened the heart as it may do in large doses on animals.[11] In three instances no primary fall of pressure was obtained (Fig. 6). It would seem that here the increased heart action was sufficient from the start to neutralize the dilatation of the blood vessels. In two instances no secondary rise of pressure occurred and the curves obtained coincide with the usually accepted theory of amyl nitrite action; viz., a rapid fall of pressure with a slow rise to the normal (Fig. 7). Such an effect would be produced by a dilatation of the arteries without a corresponding increase in heart action.

NOTES ON THE ACTION OF NITROGLYCERIN. — Widely divergent views have been expressed as to the action of nitroglycerin. Among physicians it is popularly supposed to dilate the peripheral blood vessels and to lower the blood pressure. Its toxic dose varies greatly: 1/100 grain has produced collapse and a fall of blood pressure to fifty millimeters of mercury;[12] while on the other hand, enormous doses, as much as twenty grains of the pure drug, have been taken in a day.[13] Some individuals apparently grow very tolerant to the drug and learn to take large quantities. Dr. H. P. Loomis[14] has endeavored to reduce hypertention by doses of one-fiftieth of a grain every hour, but he found that the systolic pressure was raised rather than lowered by these doses.

In Dr. J. O. Hirschfelder's wards I have frequently seen fifteen drops of the one per cent solution taken every half hour with only moderate subjective effects. In several patients a series of rapidly ascending doses of nitroglycerin were given and the dose necessary to produce decided subjective symptoms was determined. Usually this lay between five and thirty drops of a one per cent solution. On the day following, this dose was given and the blood pressures were determined subsequently every five minutes. The effect on the pressures was usually slight and inconstant. No great fall occurred. Indeed, the systolic pressure rose about as frequently as it fell. In a few patients a rise in systolic was combined with a fall in the diastolic pressure (Fig. 8). This result resembled the second stage of amyl nitrite action and a similar interpretation would assign its cause to a combined dilatation of the blood vessels and increased systolic output from the heart. When, as not infrequently happens, nitroglycerin causes the pulse to become "fuller and more bounding," I am inclined to believe that we are dealing with a similar action. The exact action of nitroglycerin in relieving certain headaches and in reducing the number of attacks of angina pectoris is less simple than is generally supposed, and it is possible that it produces its effects in these conditions, not by reducing the blood pressure but by increasing the circulation of the blood.

[I am indebted to Dr. Lehmann of Cooper Medical College for screen examinations on three patients during amyl nitrate inhalations.]

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DESCRIPTION OF FIGURES.

FIG. 1. Plethysmographic tracing from elevated hand during amyl nitrite inhalation. A rise in the curve indicates a shrinkage of the hand. Time is indicated in minutes. Duration of inhalation indicated by the straight line below.

FIG. 2. Typical amyl nitrite effect. The curves above were taken before the inhalation, those below after. Duration of inhalation is indicated by the scratched horizontal line below the lower time-marker. The latter indicates seconds and the minutes (Roman numerals) are calculated. The systolic and diastolic pressures are indicated by arrows.

FIG. 3. Curves of systolic, diastolic, and pulse pressures taken from Fig. 2.

FIG. 4. Curves showing typical marked effect of amyl nitrite.

FIG. 5. Curves showing excessive fall of pressure after amyl nitrite inhalation. Note that the pulse pressure falls to less than half its original height.

FIG. 6. Curves showing an absence ot fall of systolic pressure after amyl nitrite.

FIG. 7. Curves showing absence of secondary rise of pressure. Note also that the pulse rate is not accelerated (lack of vagus tone).

FIG. 8. Curves showing effect of nitroglycerin.

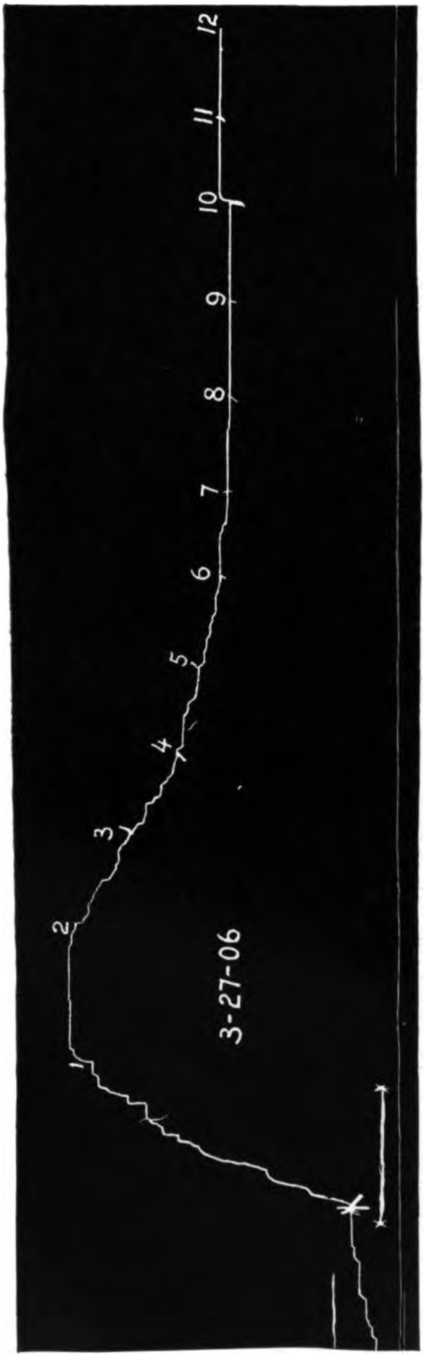

1
2
3
4
5
6
7
8
9
10
11
12
3-27-06

FIG. 1.

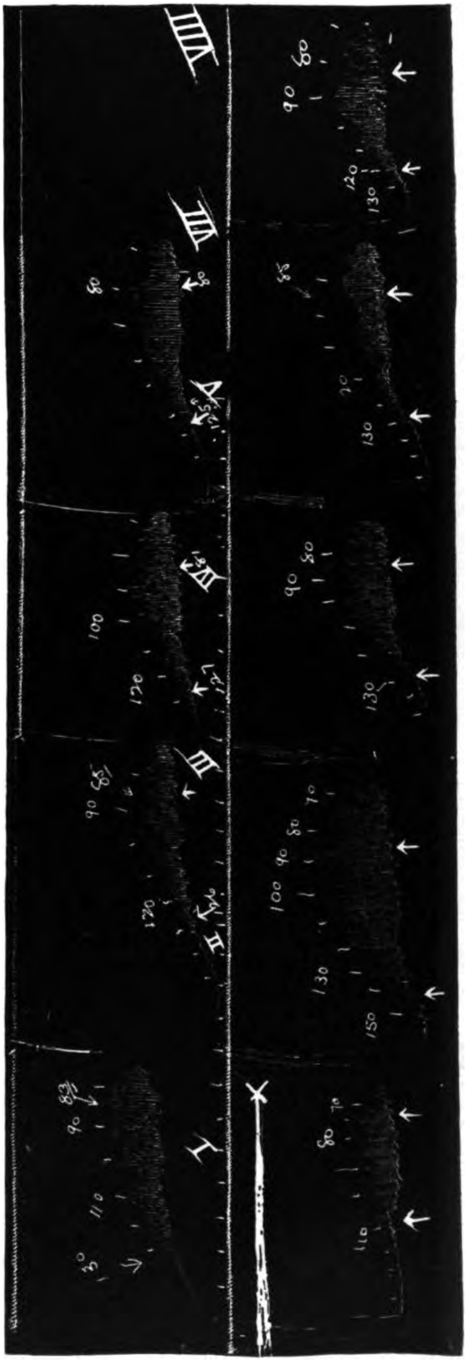
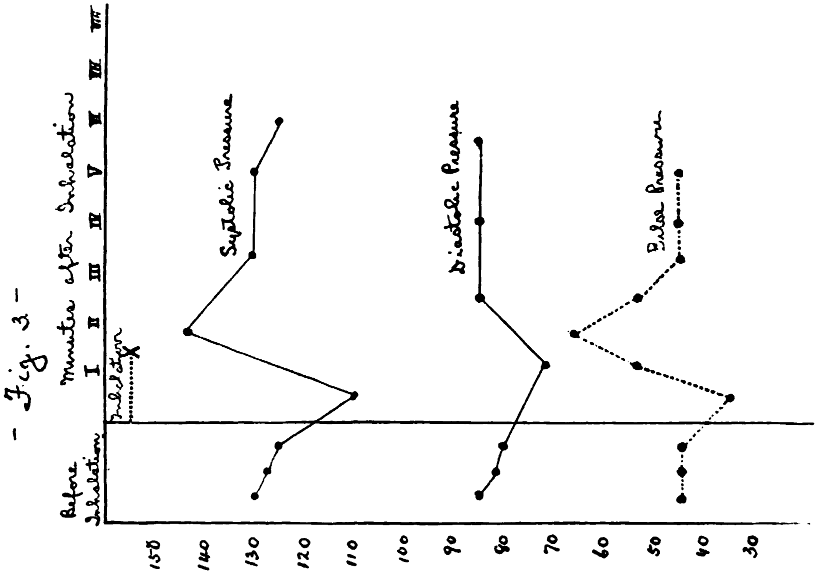
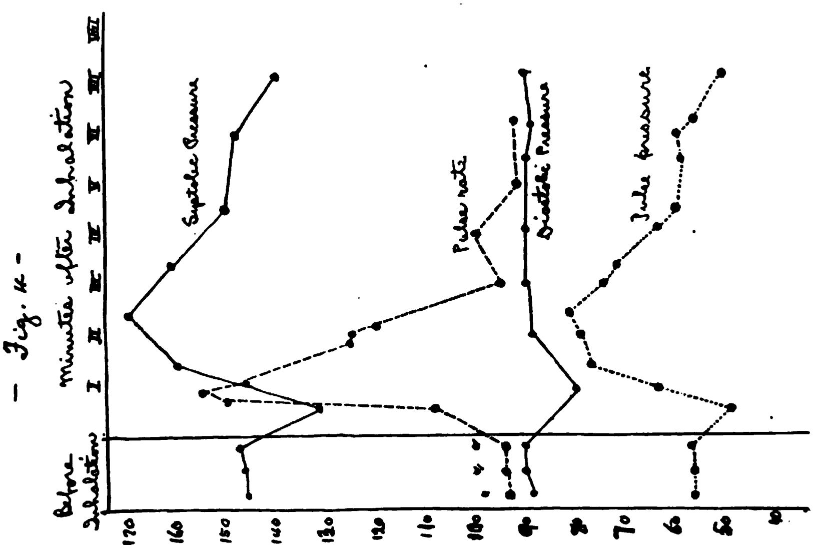

I
II
III
IV
V
VI
VII
VIII
110
90
120
100
130
150
80
70

FIG. 2.


- Fig. 3 -
Minutes after Inhalation
Before Inhalation
Inhalation
X
I
II
III
IV
V
VI
VII
VIII
Systolic Pressure
Diastolic Pressure
Pulse Pressure
150
140
130
120
110
100
90
80
70
60
50
40
30


- Fig. 4 -
Minutes after Inhalation
Before Inhalation
I
II
III
IV
V
VI
VII
VIII
Systolic Pressure
Pulse rate
Diastolic Pressure
Pulse pressure
170
160
150
140
130
120
110
100
90
80
70
60
50
40

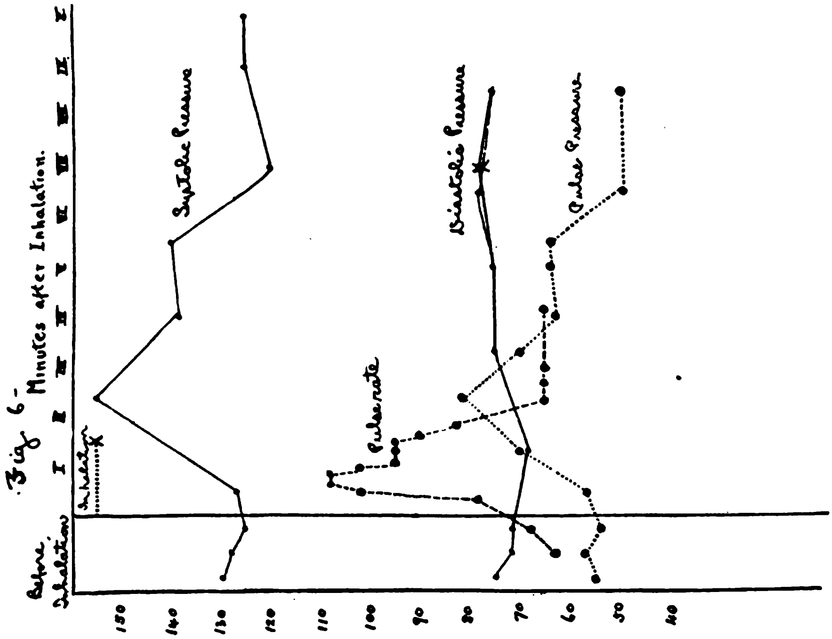
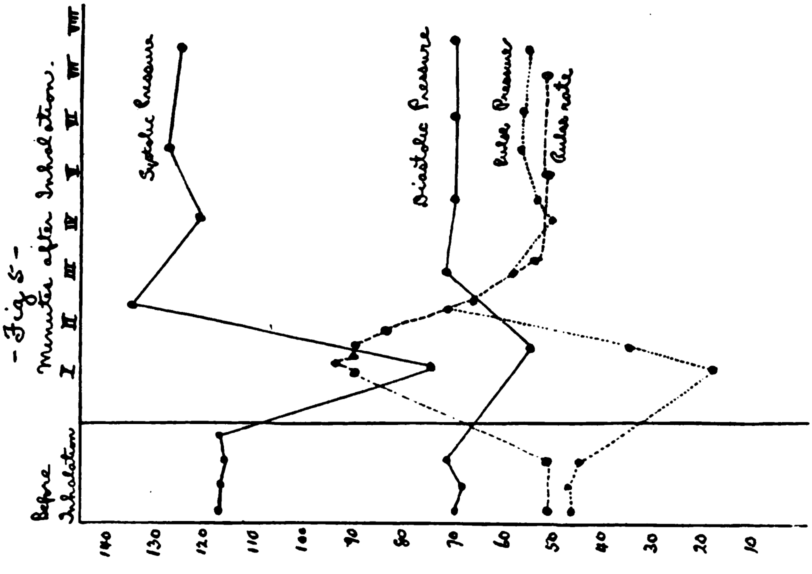


Fig 6 –
Minutes after Inhalation.
I
II
III
IV
V
VI
VII
VIII
IX
X
Before Inhalation
Inhalation X
Systolic Pressure
Pulse rate
Diastolic Pressure
Pulse Pressure
150
140
130
120
110
100
90
80
70
60
50
40


– Fig 5 –
Minutes after Inhalation.
I
II
III
IV
V
VI
VII
VIII
Before Inhalation
Systolic Pressure
Diastolic Pressure
Pulse Pressure
Pulse rate
140
130
120
110
100
90
80
70
60
50
40
30
20
10

- Fig. 8 -

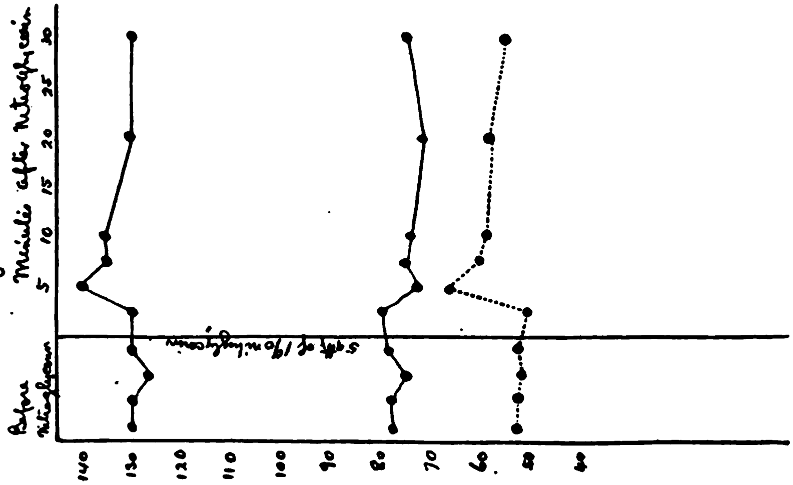
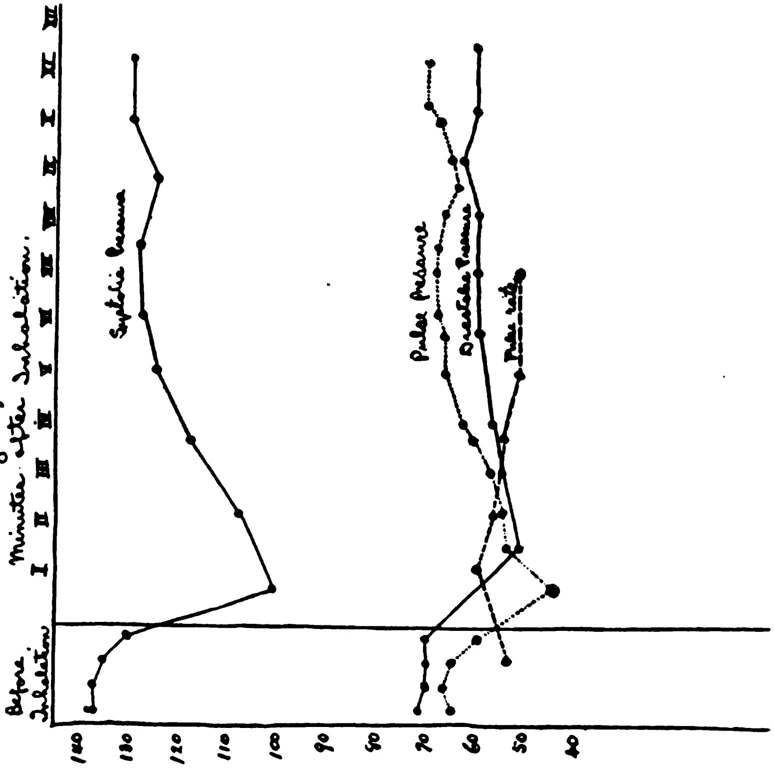

Minutes after Nitroglycerin
5
10
15
20
25
30
Before Nitroglycerin
5 gtt. of 1% nitroglycerin
140
130
120
110
100
90
80
70
60
50
40

Fig. 7


Minutes after Inhalation.
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
Before Inhalation
Systolic Pressure
Pulse Pressure
Diastolic Pressure
Pulse rate
140
130
120
110
100
90
80
70
60
50
40

A STUDY OF THE PARATHYROID GLANDULES IN PARALYSIS AGITANS.*

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It has been suggested that paralysis agitans is due to insufficiency of the parathyroid glandules. To see whether or not there is morphological ground for such a hypothesis is the main purpose of the following article.

The interest that the parathyroid glandules have aroused of late, especially as to their possible use therapeutically, may make a somewhat detailed review of our present knowledge of their history, structure, and function advisable, in view of the fact that these bodies are in general too little known.

Sandstroem,[1] in 1880, was the first to describe bodies definitely, to which he gave the name "glandulæ parathyroideæ." He found these bodies present in man and other mammalia, situated on the posterior surface of the lateral lobe of the thyroid, usually two on either side, one above and one below the inferior thyroid artery, and somewhat separated from the thyroid by connective tissue. These bodies showed a somewhat varied structure microscopically, but could be made to conform to three main types: (1), a continuous mass of epithelial cells penetrated by a considerable capillary network, unaccompanied by connective tissue; (2), a continuous cellular reticulum, the meshes of which were occupied by blood vessels and connective tissue; (3), numerous small follicles in a connective tissue stroma. This paper of Sandstroem is so thorough that little has been added to our knowledge of the anatomy or histology of these glands since their discovery, and it is so complete as to leave no doubt that he deserves all credit for the discovery of these organs.

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The completeness of Sandstroem's work is especially interesting since these bodies did not escape the eye of Virchow,[2] who remarked the frequency with which small rounded bodies, about the size of a pea, were found in the loose connective tissue on the posterior surface of the lateral thyroid lobe. But Virchow passed these by as probable lymph nodes, or detached portions of thyroid tissue. So also did Kadyi,[3] Maselung,[4] and many others. Baber,[5] at about the same time as Sandstroem, described in animals what he termed "undeveloped portions" of the thyroid gland, which were undoubtedly parathyroids, but he failed to recognize their constant occurrence or significance.

Rogowitz [6] also described what he called "restes embryonaires" in the thyroid glands of animals. He considered them, however, as parts of the thyroid in process of development. Christiani,[7] in 1893, examined the parathyroids of rodents, finding only one glandule on each side. This author also considered these organs as portions of embryonic thyroid tissue. Zielenska [8] described what were undoubtedly these glands in the dog, and considered the tissue a sort of reserve material that could furnish fresh thyroid follicles when necessary, and Hürthle [9] in a study of thyroid secretion also notes these bodies, referring to them as embryonic thyroid.

It was not until 1895, however, that any definite study of the parathyroids as such was made, notwithstanding the work of Sandstroem. In that year (1895) Kohn [10] made a valuable contribution to our knowledge of these organs in the cat, dog, and rabbit, showing that in these animals there were constantly present two of these glandules on each side of the thyroid. Since up to this time (as will be later referred to) experimental work had been carried on with the idea that only two, instead of four, of these glands were present in these animals, the importance of Kohn's work becomes evident. Kohn, moreover, considered the parathyroids as wholly independent structures, morphologically and functionally distinct from the thyroid gland, and preferred to give them the name of "epithelial corpuscles" rather than to call them parathyroid glandules. Kohn also distinguished the glandules as "external" and "internal," according to their relation to the lateral thyroid lobe. In a second paper, Kohn [11] continued his work on the "epithelial corpuscles" in rabbits, and made the observation that the external parathyroids were found at a higher level in young rabbits than in full grown. Schaper [12] reached conclusions as to the situation and significance of the parathyroids differing somewhat from those of Sandstroem and Kohn. His observations, however, are based on but five cases in man, a number hardly sufficient for serious consideration. It may be noted, however, that in one case he found six bodies instead of four. Müller [13] examined the parathyroid glands in twenty-two cases in man (as well as in the dog and the cat), but added nothing to our knowledge of their frequency and situation, as they were not specifically sought for, but examined only in course of work on the thyroid.

D. A. Welsh,[14] in 1898, made a most exhaustive study of the anatomy and histology of these glandules. He examined forty cases in man from infancy to old age, as well as the glandules of the ox, sheep, rabbit, cat, and ferret. Welsh found in nearly all cases in man two glands on each side, and states that when fewer are found, either the glands have escaped observation, or else there is a more or less intimate connection between two of the glands so that they appear as one mass. As to position, Welsh distinguishes the glands as "posterior superior" and "anterior inferior." Histologically he distinguishes two different types of cells in the glandules, which he calls "principal cells" and "oxyphile cells," and describes four types of arrangement for each.

Schreiber,[15] in the same year, studied the development and structure of these glandules, and reported that in twenty-five cases they were always found. Usually there were two glandules on a side, in four cases only one on a side was found; in two cases there was one on the right and three on the left side. He did not find more than four in any case. This author noted that the parathyroid glandules arose from a single symmetrical anlage, and that their multiplicity was due to a secondary cutting off of epithelial cell masses.

Benjamins[16] studied the parathyroid glandules to see if changes in these organs bore any relation to changes in the thyroid glandules, and confirmed the findings of the previous writers concerning position and number of these bodies. In twenty cases of goitre, this author found no progressive, but a variety of retrogressive, changes in the parathyroids.

Peterson[17] studied the parathyroids in one hundred cases in man, from routine autopsies, and confirmed the findings of previous authors as to the size, situation, and number of these glandules. The author did not fail to find the parathyroids in a single case. The external parathyroids he found most constantly present. The second most frequent finding was the four glands in normal position. In certain cases he found only one glandule. In some cases he found two or three glandules, one on one side, and one or two on the other.

Erdheim[18] found the parathyroid glandules present in cases of congenital absence of the thyroid. Similar cases had also been reported by Maresch[19] and Peucker.[20] Erdheim found in one of his cases eight parathyroids, four of which he characterized as accessory, and called attention to the fact that accessory parathyroids could at any time be present. The author also noted that cysts were frequent both in the parathyroid and accessory parathyroids, and that colloid was present in the glandules in most cases over thirty years old.

Rogers and Ferguson[21] examined forty-six adults and eight infants for parathyroid glandules. In twelve cases no parathyroids were found. In twenty, two were found; in four, three; in three, there were four glandules. The paucity of findings in these cases is quite remarkable, since the investigation seemed to be attended with much care. It does not at all agree with the work of previous authors, who also used the microscope to identify the glandules, or with my own findings.

It will be seen from the foregoing, that normally there are present in mammalia four bodies, situated on the posterior surface of the lateral lobes of the thyroid gland, two on either side, one above and behind the other and separated from the thyroid by connective tissue, to which the name "glandulæ parathyroideæ" has been given.

These organs may vary considerably in number, in shape and size, in color and in position. Their average diameter, as given by Welsh, is six to seven millimeters long by three to four millimeters wide, and 1.5 to five millimeters thick. Their most constant dimension is their thickness. In shape they may be oval, appearing as flat ovoid plates or disks, or they may have a flattened pyriform outline. Sometimes they are bean shaped, resembling a small lymph node, but are usually more flattened. At times they may be nearly square or rectangular. Their average weight is about .035 grams. In color they practically always show a shade of yellow, due to their fat content, but they may vary from pale (yellowish) gray white, to dark reddish brown. Their color is never exactly the same as the adjacent thyroid tissue or lymph nodules. The surface of the glandules is smooth and a delicate venous tracery is to be seen under their capsule. While the position of these glandules may vary, the variation is within certain definite limits, so that the position is fairly constant. The superior glandules are more constant in position than the inferior glandules. They are usually found, one on each side, on the posterior wall of the esophagus, at the posterior edge of the lateral thyroid lobes, midway between the upper and lower poles. The height may vary, however, as the superior glandules have been found as high as the inferior cornu of the thyroid cartilage and as low as the tenth tracheal ring. The parathyroids are usually wholly outside the thyroid, but they may be found apparently within the capsule of this organ. The inferior thyroid artery and recurrent laryngeal nerve pass up in front and internally to the glandules, and the bodies can usually be found at the entrance of the end branches of the inferior thyroid artery.

The inferior parathyroids are anterior to the upper bodies, lie always in front of the inferior thyroid artery, and are usually about opposite the lower pole of the thyroid, though they may be found as low as the tenth tracheal ring. They may be either posterior lateral or antero-lateral to the thyroid or may be at some distance from the thyroid (usually below it) imbedded in fat and areolar tissue. The fact that the inferior glandules are found less constantly than the superior is probably due to their inconstant position, and the difficulty in distinguishing them from small lymph nodes, nodules of thymus tissue, fat, and accessory thyroid nodules that may be found in this locality.

Accessory parathyroids may at times be found. Zuckerkandal[22] and Erdheim (l.c.) each report cases in which there were six glandules. I have found five in several instances. Accessory parathyroids have also been noted in the thymus.

HISTOLOGY. — The histology of the parathyroid glandules has been described in detail by the various authors previously mentioned, including Sandstroem, Kohn, Welsh, Benjamins, and Erdheim and, save for minor differences, the descriptions are fairly uniform. All authors describe two main types of cells which may be grouped as follows:

Type 1. — Comparatively small cells (somewhat larger than those of the thyroid) with relatively large nucleus which stains deeply. The cell protoplasm is colored with difficulty, but the cell border is distinct. These cells constitute the greater part of the gland tissue and are always present in every glandule, but may vary in size, shape, and intensity of protoplasmic stain, and in size and shape of the nucleus. These cells will be referred to as the "principal" cells.

Type 2. — Comparatively large cells with small deeply-staining nucleus and considerable deeply staining, eosinophilic, granular protoplasm. These cells have a distinct cell boundary. They are not found in every glandule, though they are present in almost all cases. They are never so numerous as the "principal" cells. These cells will be referred to as the "functional" cells.

The number and arrangement of these cells is subject to wide variation. They may occur in masses, forming small islets scattered throughout the gland tissue, or may occur irregularly scattered among the principal cells, either singly or in groups of three or four, without any definite arrangement. Sometimes they form definite acini or occur in continuous anastomosing columns.

The principal cells are also subject to a great variety of arrangement, giving different pictures in different glandules. The cells may form a uniform mass continuous in every direction, being broken only at infrequent intervals by delicate strands of connective tissue carrying small blood vessels. The cells in this instance appear irregularly polyhedral, and the cell walls are everywhere in direct contact with each other.

The glandules may show connective tissue, with blood vessels, between the cell masses, thus breaking them into anastomosing columns, or cell trabeculæ. Here the cell protoplasm stains more deeply and the cells are apparently rounder than in the former arrangement. There may be seen further subdivision of the cell masses by fibrillar stroma or capillary reticulum, or denser fibrous stroma with blood vessels, so that small islets of epithelial cells appear within the stroma somewhat akin to the arrangement in carcinoma.

In small parts of the glandules the "principal" cells may form definite acini, so that the structure resembles secreting gland, the center of which usually contains colloid.

The glandules present, then, in general, a together-hanging cell mass composed for the most part of small cells with indistinct protoplasm and deeply-staining nucleus, interrupted irregularly by small islets, or groups of two or three, larger eosinophilic cells, which may or may not have a characteristic arrangement. These cell masses are divided irregularly by blood vessels into lobular or reticular cell strings, or may be separated into distinct lobules by connective tissue septa. Both the cells and the connective tissue may contain considerable fat, which is believed by most authors to arise in the organ itself without outside influence.

PATHOLOGY. — Although various degenerations and other pathologic conditions have been described in the glandules, these organs cannot be said to have a distinct pathology. In Peterson's (l.c.) cases the author notes the frequency with which degenerative changes were found in the organs from cases over twenty years old. These cases consisted in atrophy of the parenchymatous cells brought about either by increase of fatty connective tissue in the glands, or by parenchymatous or cystic degeneration. Of his one hundred cases, twenty-five showed parenchymatous swelling; fifteen, colloid; six, cyst formation; twenty-one, fatty infiltration. The author concludes that there was no connection between the disease of the patient and the pathologic conditions of the glandules.

Benjamins (l.c.) found hydropic degeneration in twenty-five cases; pigment atrophy; connective tissue increase and frequent colloid. He described a tumor of the parathyroid, the size of a child's head. The presence of fat, which was constantly found, the author considered as physiological. Erdheim (l.c.) notes mast cells in the connective tissue of some of the glandules; glycogen he found frequently present. Colloid Erdheim considered as representing a return to original structure and as having nothing to do with function. Cysts of the parathyroids have been described.

MacCallum[28] showed that in certain instances these glandules exhibit greatly increased activity. In two instances glandules were found in which the granules of the "functional" cells were especially large, and in which the "principal" cells showed a rich development of mitotic figures.

PHYSIOLOGY. — These organs, which were for so many years overlooked; which are so comparatively unimportant from an anatomic standpoint; and which present so wide a variety of histologic picture as to make the idea possible that they are rudimentary rather than functional organs, assume importance when regarded from an experimental side, which makes the question of their function in the body economy one of great interest to medicine.

For many years it had been noted that certain animals after thyroidectomy showed convulsive or tetanic symptoms which quickly led to death, but no one had considered these symptoms due to anything other than absence of the thyroid gland till the time of Gley.

Gley[24] conceived the idea that these tetanic symptoms seen in animals after thyroidectomy were not due to the removal of the thyroid, but due to the removal of the parathyroid glandules. Previously to this time it was thought that this difference in susceptibility to thyroid removal was due to difference in metabolism; the animals suffering most being flesh eaters; the animals suffering least being vegetable and grain feeders (Horsley,[25] Briesacher[26]).

Gley showed, however, that in the rabbit the (external) parathyroids were quite separate from the thyroid, while in the dog they were incorporated within the thyroid lobe. Therefore, thyroidectomy had not involved a loss of the parathyroid in the rabbit, while it had in the dog.

In support of his theory Gley removed both the thyroids and the parathyroids from sixteen rabbits. Fourteen of these animals following such treatment showed rapidly fatal symptoms, which began within twenty-four hours after operation and led to death in a few hours. In all, Gley removed the external parathyroids in fifty-five rabbits, only twelve of which survived. (It is to be noted that the internal parathyroids, as well as the islets of parathyroid tissue in the thymus, were then unknown.)

Gley also showed that in the dog if the thyroid was removed and the parathyroids were left intact, the animal did not show acute symptoms, while if the parathyroids were subsequently removed the usual fatal termination resulted. Similar results were obtained in the rabbit. When the parathyroids were removed a month subsequent to the thyroid removal, the animal died with acute symptoms and the parathyroids were found to have undergone hypertrophy.

The work of Gley was supplemented and confirmed by the experiments of Hofmeister,[27] Christiani,[28] Verstraeten and Vanderlinden,[29] Cadeac and Guinard,[30] Paladino,[31] Edmunds,[32] and Rouxeau.[33]

The results of Moussu[34] differed considerably from the above, as he claimed to have had only four deaths in nineteen rabbits from which thyroid and parathyroids were removed. The paper of Blumreich and Jacoby[35] also assailed Gley's conclusions.

The reason for these conflicting results could in a measure be accounted for by the difficulties of technic, but the experimental work was soon placed on a new and better basis by the discovery of Kohn of the "internal" parathyroids.

After the publication of Kohn's paper (l.c.) establishing the presence of the "internal" as well as the "external" parathyroids, Vassale and Generali[36] removed all four parathyroids in dogs and cats, leaving the thyroid in situ, and obtained in all cases fibrillary tremors, muscular twitchings, depression, rigid and staggering gait, emaciation and death. Later papers by Rouxeau[37] and Moussu[38] did not, however, contain such uniform results.

Welsh (l.c.) removed the four parathyroids in the cat, and obtained severe and fatal symptoms; even with one parathyroid left in situ results were similar. If two parathyroids were left no serious acute symptoms resulted. The administration of ox parathyroids by the mouth did not relieve the symptoms.

Gley,[39] in a later paper, referring to these first experiments (Vassale and Generali, Rouxeau, Moussu and himself) which led to the inference that the thyroid and parathyroids had absolutely distinct functions, says: "This conclusion was premature. I have noticed two cases where the dogs survived after extirpation of all the glands (parathyroid), a single lobule of the thyroid being left. On the other hand, there was in two other animals after the same operation a slow development of disturbances, which became grave after the ablation of the remaining lobe of the thyroid, and then caused death. I have seen the same thing in the cat." In dogs and rabbits he had sometimes observed only nutritive disturbances after parathyroidectomy.

Edmunds[40] also reports two cases of survival of dogs after parathyroidectomy.

MacCallum[41] attempted to produce a specific cytotoxin for the thyroid alone, and for the parathyroid alone, and to compare their results. Parathyroids of dogs were therefore injected into the peritoneal cavities of geese. Blood from the wing vessels of geese so injected was introduced into fresh dogs. This caused symptoms of emaciation in the dogs. One dog showed convulsive movements, but not similar to those occurring after removal of the parathyroids. No injury was caused to the thyroid cells or to the parathyroids following the injections. The author gives a graphic description of the tremors, tetanic spasms and convulsions occurring in dogs after parathyroidectomy.

Pineles[42] obtained after thyroidectomy various myxedema symptoms but neither tetany, spasms, nor paralysis, if the parathyroids were left intact. Experiments on cats and dogs, as well as on apes, showed certainly a connection between tetany and parathyroid removal. The author concludes, "that tetania strumipriva of man, and tetania thyroipriva of animals, is rightly to be named tetany after parathyroid removal."

Quite recently a very convincing communication has been made by Erdheim[43] on the tetany of parathyroid insufficiency. Erdheim removed the parathyroids in fifty rats, wholly or in part. When the parathyroids were wholly removed, tetany followed, and the animal died of cachexia in two to five and a half months; if thyroidectomy was performed, but the parathyroids left intact, there was no tetany. If parts of the parathyroids were excised, the frequency of tetany bore a direct ratio to the amount of parathyroid tissue removed. Results in rabbits were not so certain, and Erdheim accounts for the lack of tetany at times in these animals, following parathyroid removal, by the fact that rabbits show numerous islets of parathyroid tissue in the thymus. These "accessory" parathyroids may be sufficient for function even after removal of all four epithelial corpuscles.

The author also examined three cases in men dying of tetany after partial double thyroidectomy, and showed that in these cases in which some of the thyroid was left behind, the parathyroid tissue had been completely removed. The loss of parathyroids, then, and not the loss of thyroid tissue, the author concludes to be the cause of tetany following these neck operations.

THE SUPPOSED RELATION BETWEEN INSUFFICIENCY OF THE PARATHYROIDS AND PARALYSIS AGITANS. — The experimental work that has been done in the removal of the parathyroid glandules in animals, notwithstanding certain minor conflicting results, is too thorough, in general, to admit of any doubt but that in most instances where these organs are wholly removed the animals died from acute, rapidly fatal, nervous phenomena, including various degrees of tetany; whereas if only the thyroid gland is removed, and the parathyroids left behind, there appears slowly a cachectic condition (myxedema). In other words, as epitomized by Jeandelize,[44] "Insufficiency of the thyroid causes chronic troubles (nutrition), while insufficiency of parathyroids causes 'acute troubles' (convulsive troubles)." When these results became known, the relation of parathyroid insufficiency to diseases in which there were tetanic symptoms at once suggested itself. Moussu[45] formulated the theory that exophthalmic goitre depended on parathyroid insufficiency. Gley[46] suggested that "exophthalmic goitre is due to an alteration in the thyroid apparatus involving in the first place the parathyroids." Edmunds,[47] at the same time, suggested partial aparathyroidia as an explanation of exophthalmic goitre, Benjamins (l.c.) and others, however, found the parathyroids normal in exophthalmic goitre, and this theory was not supported.

Other convulsion troubles, *e.g.*, epilepsy, infantile convulsions, eclampsia and tetany have been suggested by various authors, including Moussu (l.c.), Jeandelize (l.c.), Luzena (l.c.), Vassale and Generali (l.c.), and later MacCallum (l.c.), as being due to an insufficiency of the parathyroids. Lundborg[48] suggests that the so-called "Arbeitertetanie," as, for example, in tailors and shoemakers, who work all day with necks bent forward, is due to an insufficiency of the parathyroids brought about by the disturbed circulation in these organs incident to the cramped neck.

It is to Lundborg (l.c.), however, that we owe the hypothesis that paralysis agitans is due to insufficiency of the parathyroid glandules. Berkeley[49] conceived the same idea independently, although his paper was not published till some months later.

It is quite remarkable that this suggestion should not have come earlier, and more generally, since nearly twenty years ago the following statement was made by Horsley[50] in connection with thyroidectomized apes. (The description of his operation leaves no doubt that the parathyroids were also removed, but at that time little or no attention had been paid to these organs.) He says: "In the monkey I have found that, as a rule, the animal, after complete thyroidectomy, appears perfectly well for about five days. Then there is noticed a slight fibrillation of the intrinsic muscles of the hands, feet, and jaws following this order in successive invasion. As a rule the fibrillation soon becomes a constant tremor. The constant tremor is soon added to by a series of powerful clonic spasms. This paroxysmal stage usually appears about the second or third day after the tremors are first noted, and persists about twenty days. They gradually fall in force, reassume the type seen at their onset and disappear sometimes as much as ten days before death. A cursory observation shows the subject to be one of immense importance, as tending to explain, not the pathology of occasional fits of trembling, but what is of infinitely more importance, the causation of a constant tremor such as in paralysis agitans." Another suggestive picture of a thyroidectomized ape is given by Langhans,[51] who thus described the animal: "Ich habe die Tiere vielfach gesehen, wo sie hülflos auf dem boden sassen, zum theil mit Zwangsbewegungen, namentlich des korpes and selbst des ganzen körpers nach links, ein rechtes mitleid erregendes Bild."

These pictures have, however, apparently attracted no especial attention as far as paralysis agitans is concerned, and form no bases for Lundborg's hypothesis that this disease is a chronic, progressive, hypoparathyroidismus.

Lundborg's paper, as well as Berkeley's, is wholly hypothetical, and this present work was undertaken to see definitely whether there was morphologic reason for the hypothesis of these authors.

Lundborg (l.c.) cites Luzzato, Dana, Möbius, Frenkel, Burzio, Casteloi, and Schiefferdecker, all as expressing the idea that the disease is an endogenous toxemia. He emphasizes the tetanic symptoms that arise in animals after parathyroidectomy; and after a considerable discussion, which is impossible to completely abstract, gives a diagram wherein the system of thyroid and parathyroid hypo and hyper function is delineated in terms of myxedema, morbus Basdowii, paralysis agitans, and paralysis myasthenia respectively. Lundborg's paper must be read in its entirety to appreciate the full force of his arguments. He concludes with the statement, however, "das es aber noch für ausserst hypotetish gehalten werden muss, ob die glandulæ parathyroidæ eine bestimmte Rolle in deren Pathologenese spielen."

Berkeley (l.c.), who has independently brought forward the idea that "atrophy or insufficiency of the parathyroid glands is perhaps the cause of paralysis agitans," cites in support of his theory: the tetanic symptoms observed in slowly dying parathyroidectomized rabbits; the endogenous toxemia concept of the disease, which seems to be borne out in a measure by the fact that the author considered that the administration of parathyroid gland extract was of therapeutic value in a majority of his cases; and the possibility that, in cases of concurrent myxedema and paralysis agitans the parathyroids may have been diseased or atrophied through contiguity with the diseased thyroid. Berkeley was unable, up to the time his paper was written, to obtain post-mortems on any case of paralysis agitans, and therefore states that the final disposition of his proposition must rest on autopsy findings.

I was fortunate enough to obtain the material from post-mortems on nine cases of paralysis agitans during the past spring and summer, and the following work is the result of the examination of the same, to which is added the control study of parathyroids in ordinary diseases.

Control material: In forty routine autopsies (not paralysis agitans) the neck organs were examined for parathyroid glandules. In every instance frozen sections were made of the tissue removed, and all bodies that are designated as parathyroids represent a diagnosis made with the microscope of permanent stained sections. A great many pieces of tissue were cut and stained on the possible chance of their being parathyroid glands, which proved instead to be lymph nodules, thyroid, thymus, fat, sympathetic nerve ganglia and other tissues. In thirty-three of the forty cases, four glandules were found. Three of these cases presented also accessory glandules (five instead of four in each instance). In four of the forty cases only three glandules were found; in one instance one of the superior glandules was missing, in the other cases it was one of the inferior glandules that was not found. In two cases, only the two superior parathyroids were found; the inferior glandules were missing. In one case no parathyroids were found.

The identification of these glandules in general is not difficult after a little practice, but sometimes they are found only after a most tedious and painstaking search. Frequently, when I failed to find the bodies in their usual situation at autopsy, I placed the neck organs in ten per cent formalin solution and examined them the following day. This hardening of the tissues, especially if there is much edema and congestion of the neck, aids in finding glandules that could be overlooked in fresh material. In nearly all of my cases where I did not find four parathyroids there was some pathologic condition present that made the search difficult. Two cases were subsequent to carcinoma operation; one showed extensive tuberculous cervical lymphnoditis, and in others the neck organs were edematous and congested. With but one or two exceptions where I failed to find four bodies I felt that I had in some way overlooked the glandules that should have been present.

The case in which no parathyroid glandules were found, contrary to the above, presented every facility for search. The neck organs were from a woman seventy years old, who died from cerebral hemorrhage. She had marked arthritis deformans, and was much emaciated. The thyroid gland was atrophied to less than one-half the normal size. There was nothing to obscure the parathyroids had they been present. Serial sections were made of what connective tissue there was in the neighborhood, and of the lateral thyroid lobules, but no trace of parathyroid tissue was found. It seems fair in this case, then, to say that there were no parathyroid glandules present.

Regarding the size, shape, color, and position of the parathyroid glandules in these cases I can add nothing to what has previously been written. The position of the upper glandules I found most constant. They were usually to be found midway between the upper and lower poles of the lateral thyroid lobe just at the bifurcation of the end branches of the inferior thyroid artery. They were usually separated from the thyroid, but sometimes lay directly upon it, separated only by a thin fibrous capsule. Sometimes I have found the glandules opposite the upper pole of the thyroid and quite anterior to their normal situation.

While the inferior glandules are not so constant in position as the superior, they can generally be found at about the level of the lower pole of the thyroid, internal to the inferior thyroid artery and usually lying free in the fat and areolar tissue. They may lie on the thyroid. Quite frequently they are found on the anterior, rather than the posterior tracheal surface. I have found them sometimes occupying nearly the place of the superior glandules, all four parathyroids lying close together. I have also found them several centimeters below the thyroid lobe, and lying on the anterior tracheal surface.

There is little to be added to what has already been written on the histology of these glands. The various "types" of cell arrangement that have been described may be found, but I see no reason for such division into various types, any more than that we should find "types" for the different pictures we get in the spleen under various conditions that are not considered pathological.

The varying amount of connective tissue in the gland is notable and gives a wide variety of picture. The ratio between "principal" cells and "functional" cells is also at wide variance. All of the glandules contained fat in varying amounts. Sometimes all the cells were fairly loaded with fine and coarse fat granules. Frequently the connective tissue of the gland was very fatty. Colloid was not an infrequent finding. Small cysts were frequently found; sometimes a considerable number occurring in a single glandule. The protoplasm of both types of cells frequently showed degeneration. These degeneration products (glycogen, fat droplets, etc.) frequently push the cytoplasm to the edge of the cell, giving an optical appearance of intercellular framework, which Pick[52] had noted in adrenal tumors. This line of cytoplasm Peterson (l.c.) holds, wrongly I believe, as a cell secretion lying in the space between the cells.

THE CASES OF PARALYSIS AGITANS. — For the study of parathyroid glandules in this disease the neck organs were removed entire and the glandules in some instances dissected out, immediately, and fixed both in ten per cent formalin and in Zenker's fluid. In other cases the neck organs were placed in formalin entire and the glandules dissected out after fixation. Both paraffin and frozen sections were made of the glandules, and hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin, Van Gieson, and Scharlach R. staining were employed.

Case I. (Plate XXVI., Fig. 1). — Shows four parathyroids in normal situation. The superior glandules measure 4 x 2 x 1.5 millimeters respectively. The inferior measure 9 x 5 x 0.5 millimeters and 8 x 5 x 0.5 millimeters. All are free from the thyroid and lie in the fat tissue. The right upper glandule is divided by a connective tissue septum into two distinct lobes.

Case II. (Plate XXVI., Fig. 2). — In this case five parathyroids are found. The superior glandules are smaller than usual. They measure respectively 2 x 1 x 1 millimeters and 3 x 2 x 1 millimeters. The left is 1 centimeter higher than the right. The inferior glandule on the right measures 7 x 5 x 1 millimeters. On the left side are two glandules, just below the lower pole of the thyroid, posterior. One is crescent shaped and curves around the lower pole of the thyroid, measuring 10 x 2 x 0.5 millimeters. The other is oval and a trifle larger than the superior glandules. The left upper parathyroid is situated beneath the capsule of the thyroid gland. The others are free.

Case III. (Plate XXVI., Fig. 3). — In removing the neck organs the cut was made so high that a portion of the left lateral thyroid lobe was left behind, thus losing the chance of finding the inferior parathyroid on that side. The other three glandules were found. The superior glandules are in normal position and are of practically the same size, measuring 5 x 3 x 0.5 millimeters. The left is constricted in the center by a fibrous band. The inferior parathyroid on the right is larger than usual with one end tapering, the other rounded, and measures 8 x 4 x 3 to 2 millimeters. The glandules are free from the thyroid gland.

Case IV. (Plate XXVII., Figs. 5 and 6). — There are four parathyroids present. The left upper glandule is higher than the right and lies at the upper pole of the thyroid 3 centimeters above the last branch of the inferior thyroid artery. The right upper glandule is in normal situation. The inferior parathyroids lie on the tracheal surface of the thyroid in the anterior segment of the gland. The left is just anterior to the lateral edge of the lower pole of the thyroid. The right is a bit lower and 1 centimeter from the anterior median line. All of the parathyroid glandules in this case are somewhat lenticular in shape and of similar size, measuring 5 x 2 to 3 x 1 to 1.5 millimeters. They are free from the thyroid.

Case V. (Plate XXVI., Fig. 4). — The thyroid is much atrophied, the lateral lobes measuring only 3 x 2 x 0.5 centimeters. The superior parathyroids are in normal situation, but are quite small, measuring 5 x 1.5 x 5 millimeters. They are lenticular in shape. The right internal parathyroid is of normal size and situated just posterior to the lower lateral lobe of the thyroid. The left internal glandule is not to be found.

Case VI. (Plate XXVIII., Fig. 1). — Only three parathyroids are found. The upper glandules are in normal position. The left measures 5 x 4 x 1.5 millimeters. The right is larger and flatter and measures 1 centimeter x 6 millimeters x 0.75 millimeter. On the right side, at the base of the lower lateral edge of the thyroid, is a large cyst 2.5 x 2 x 2 centimeters, at the inner pole of which the parathyroid glandule appears as a flattened cap. No glandule was found on the left lower side.

Case VII. (Plate XXVII., Fig. 7). — The thyroid in this case is also quite small. The lateral lobe measures 4 x 2 x 1 centimeters. Four parathyroids are found. The left superior parathyroid measures 10 x 4 x 0.75 to 1 millimeter. It is situated considerably lower than usual (close to the inferior glandule) and is covered by the inferior thyroid artery. The right upper glandule is in normal situation. It is quite flat and measures 6 x 4 x 0.5 millimeters. Close to it is a very small accessory parathyroid, separate, but in close contact, measuring 3 x 1 x 0.5 millimeters. The right inferior glandules are symmetrically situated on the lower pole of the thyroid. They are roughly oval and measure 5 x 6 x 1 millimeters.

Case VIII. (Plate XXVII., Fig. 8). — All four parathyroids are present and in normal position. The left upper is oval; 5 x 4 x 1 millimeters. The right upper is symmetrical with left, but small and lenticular in shape; 4 x 2 x 1 millimeters. The right inferior glandule is half way between the middle of the thyroid and the lower pole, on the posterior surface of organ, and measures 8 x 5 x 2 millimeters. The left inferior is a trifle lower than the right, measures 10 x 4 x 2 millimeters, and possesses a tail-like process 4 millimeters long running back behind the edge of the gland.

MICROSCOPIC: Case I. (Plate XXVIII., Fig. 2). — The glandules contain a moderate amount of connective tissue, so that a somewhat, though not marked, lobular structure is apparent. The lobules vary in size, and are made up of closely set small cells. The nuclei of these cells are round or oval and stain deeply. The protoplasm stains poorly. The cell borders are usually distinct. These cells are comparatively small with a relatively large nucleus, but vary in size, shape, and intensity of nuclear stain. These cells lie most everywhere in close contact with each other, without any definite glandular grouping, but occasionally they form definite acini. Capillaries filled with red blood corpuscles are frequently seen between the cells. Occasionally large cells with granular protoplasm are seen irregularly scattered among these cells previously described. Usually these occur singly, but sometimes a group of two or three are seen. Mast cells are seen in the connective tissue. With the Scharlach R. stain, the cells are found to be filled everywhere with fine and medium sized fat droplets. There is practically no fat in the intercellular stroma.

Case II. — The structure of the glandules in this case is somewhat similar to that in Case I., but there is a greater amount of a delicate connective tissue stroma between the cell masses giving them a more definite grouping in places, so that the general picture is somewhat similar to that of the adrenal. Definite acini are seen in places holding small round colloid masses in the center. One small islet and occasional isolated cells of the large granular type are seen. There is a considerable amount of fat in fairly large droplets quite generally present in the cells.

Case III. — The structure of the glandules is not greatly different from Cases I. and II. There is very little connective tissue stroma. For the most part the cells are closely set. In places there is a strand-like arrangement. The capillaries and small blood vessels are markedly injected. There are occasional small islets where the parenchyma of the gland is replaced by fatty tissue. One of the glands shows several good sized islets of the "functional cells" and there are many single cells of this type scattered throughout the gland. All the cells contain considerable fat in large and small droplets.

Case IV. — In this case the glandules show a moderate connective tissue stroma, bearing blood vessels, which breaks the glands into small islets of epithelial cells which have no particular style of arrangement. Different groups of the cells show relatively different amounts of protoplasm, *e.g.*, one cell group will consist of a mass of closely set nuclei with little or no protoplasm or cell membrane, while a neighboring cell group will show similar shaped and sized nuclei occupying the center of cells with a considerable poorly stained protoplasm and distinct cell membrane. There is only an occasional large granular cell to be made out. The glands show quite large islets of fatty connective tissue replacing epithelial tissue, and the epithelial cells everywhere contain fat in moderately large droplets.

Case V. (Plate XXVIII., Fig. 3). — The upper glands show a marked connective tissue stroma, which contains considerable fat, dividing the cell masses into small islets so that we have a structure comparable with the arrangement in carcinoma. The cells in these islets are in closely set masses and show but little protoplasm. One islet of the functional cells is seen. The blood vessels are injected. The epithelial cells show fat in moderate sized and small droplets.

Case VI. — The superior glandules show a moderate connective tissue stroma enclosing typical closely set cell masses continuous in every direction. In the right inferior glandule "functional" cells are quite common; several can be seen in each oil immersion field, and several islets of these cells are scattered throughout the section. The granules of these cells are sometimes quite large and stain deeply with the eosin (Plate XXVIII., Fig. 4). The cells everywhere show considerable fat.

Case VII. — The glandules appear similar to Case I. (closely set cell masses). One of the glands shows replacement of about half the parenchyma with fatty connective tissue. There is a considerable amount of fat present in the cells in fine droplets.

Case VIII. — The glandules in this case show nothing that differs from the cases previously described. All of the glandules show islets of the "functional" cells and also single cells of this type irregularly scattered. There is a moderate amount of connective tissue present. Fat is seen quite generally both as fine and medium sized droplets in the cells and in the connective tissue.

Case IX.* — This case shows four normally situated parathyroids, and one accessory glandule. The upper glandules measure 8 x 4 x 1 millimeters and 10 x 3 x 1.5 millimeters respectively. The inferior glandules lie on the lateral surface of the thyroid lobes, at the lower pole, closely applied to the thyroid. The right measures 12 x 4 x 0.5 millimeters. The left measures 7 x 3 x 1 millimeters. There is a small accessory glandule on the right 3 x 1 x 0.5 millimeters.

Microscopically the glandules are of the normal type, the glandule being made up of closely set cell masses of the "principal" cells in which are small islets of the "functional" cells. There is a delicate connective tissue stroma separating the cell masses into definite lobules. In some places the cells form fairly definite acini. Fat is present in only moderate amount.

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* My ninth case of paralysis agitans was obtained for me from the St. Louis Female Hospital by Dr. D. L. Harris, to whom I wish to express my thanks for the same.

SUMMARY. — In summing up these nine cases of paralysis agitans there is little to be said save that the parathyroid glandules, in individuals dying with this disease, present no changes either in number, size, position, or histologic structure that would serve to distinguish them from the parathyroid glandules in individuals dying from other diseases.

In two of the nine cases, only three glandules were found (in one other case where only three glandules were found the technic was faulty). Many writers find three glandules more often than they find four, in routine examination. In three cases five glandules were found. The finding of the minute accessory parathyroids in these cases more frequently than in the routine cases was undoubtedly due to the extra care that was used in searching for them in the paralysis agitans material.

The average size of the glandules was a little smaller in the cases of paralysis agitans than the general average of the routine cases. This can be accounted for by the fact that all the paralysis agitans cases were over seventy years of age. Routine cases over seventy years of age show in general smaller glandules than young individuals.

There was nothing to be found microscopically in the parathyroids in the paralysis agitans cases that could differentiate them from other parathyroids. Five of the cases can be passed over without remark; they were in every way identical with the type picture presented by the majority of the glandules in routine parathyroid examination. Two of the cases presented a moderate increase of connective tissue. The percentage of glandules showing a considerable connective tissue stroma was higher in the routine cases than in paralysis agitans. Eight of the routine cases were of this type. One of the paralysis agitans cases showed a great amount of interlobular fatty connective tissue, so that only small islets of glandular tissue were seen. Three of the routine cases, however, gave even greater connective tissue stroma, with corresponding atrophy of the parenchyma. Fat was found in every glandule examined, both in the paralysis agitans and the routine cases. Some of the paralysis agitans cases showed a great amount of fat. Many of the routine cases showed an equal amount, and some even more. The glandule that showed the most fat was from a case of diabetes.

All of the cases of paralysis agitans showed both types of cells ("principal" and "functional"), although the ratio between the two varied greatly in different cases and in different glandules from the same case, as they did also in the routine cases. In one case, one of the inferior glandules exhibited apparent increased activity of the "functional" cells. No mitotic figures were seen in the "principal" cells, either in the paralysis agitans or routine cases.

In conclusion, then, it must be stated that there is no morphologic ground for the assumption that the parathyroid glandules are insufficient in paralysis agitans. Whether or not there is faulty secretion of these organs; whether they are unable to cope with a poison circulating in the blood that their cells or their secretion is not able to neutralize; or whether the specific relation between these glandules and some other organ or organs is upset, must be determined by experimentation other than morphologic, and offers a promising field for investigation. On morphologic grounds there is every reason to oppose the hypothesis that paralysis agitans is a "chronic, progressive hypoparathyroidismus."

[The work was undertaken at the suggestion of Dr. L. Pick, and carried on under his supervision in his laboratory at the Landau clinic. The autopsies on the cases of paralysis agitans were done at the Friederich Wilhelm Hospital, by permission of Sanitætsrat, Dr. Wilhelm Græffner, and the material for the control cases was obtained at the Jüdische Krankenhaus from the routine autopsies done by Dr. Pick. For the kindness of Dr. Pick and his associates, which has made this work possible, I wish to express my most sincere thanks.]

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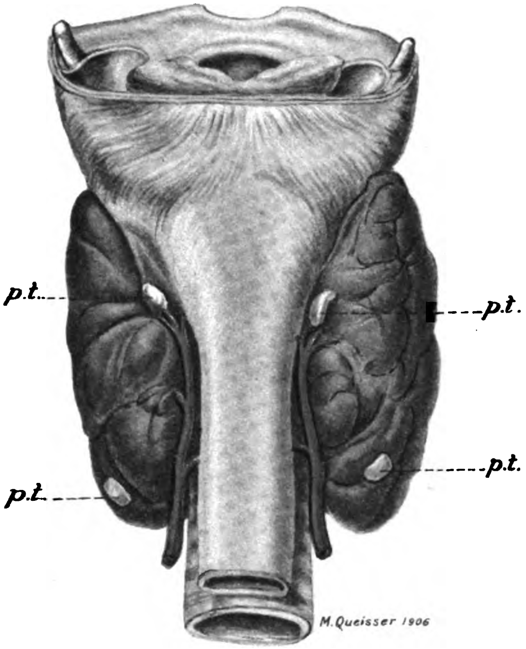
DESCRIPTION OF PLATES.

PLATES XXVI. and XXVII., Figs. 1 to 8, and PLATE XXVIII., Fig. 1. — Sketches of neck organs showing situation, size, shape, and number of the parathyroid glandules (p. t.) in the first eight cases of paralysis agitans. Line aa, Fig. 3, shows high cut in removal of the neck organs so that the lower part of the thyroid lobes were left behind.

PLATE XXVIII., Fig. 2. — Paralysis agitans. Case 1. Lobules of "principal" cells, separated by moderate connective tissue stroma. Fat droplets in the cells stained red with Scharlach. x 400.

Fig. 3. — Paralysis agitans. Case 5. Considerable connective tissue stroma (in places fatty), separating the "principal" cells into small islets. A fairly large islet of "functional" cells in the lower right part of section. Fat is stained red. x 65.

Fig. 4. — Paralysis agitans. Case 6. Islet of "functional" cells and single cells of this type among "principal" cells. The functional cells are large and their cytoplasm stains deeply with the eosin. The principal cells are small and their cytoplasm is practically unstained. x 575.



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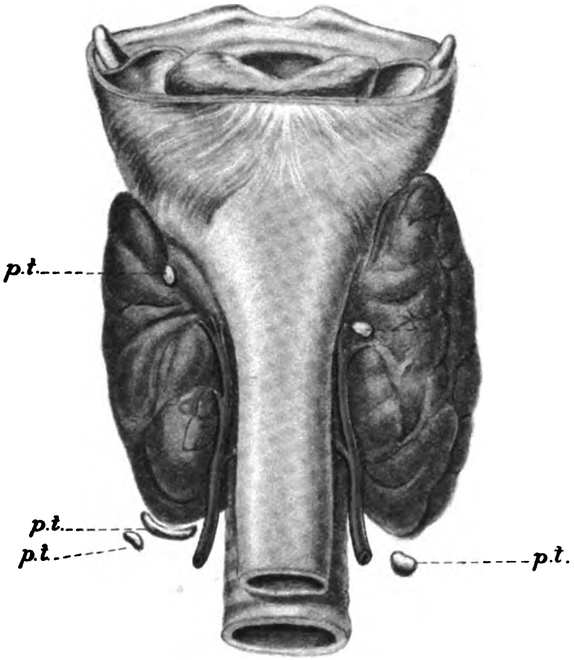

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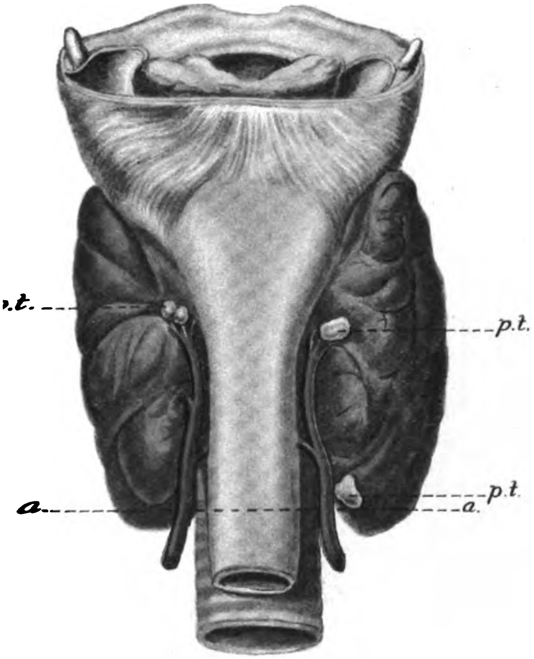

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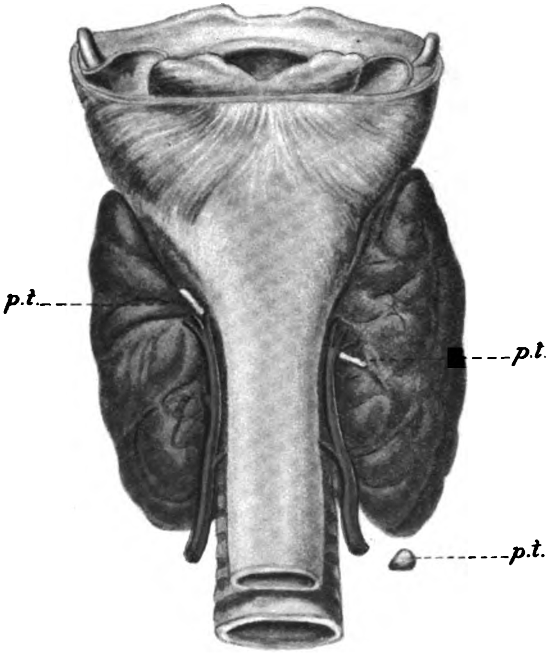

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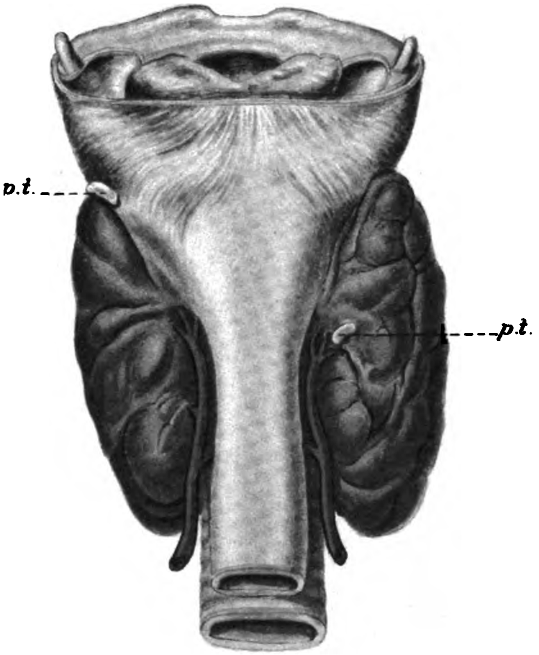
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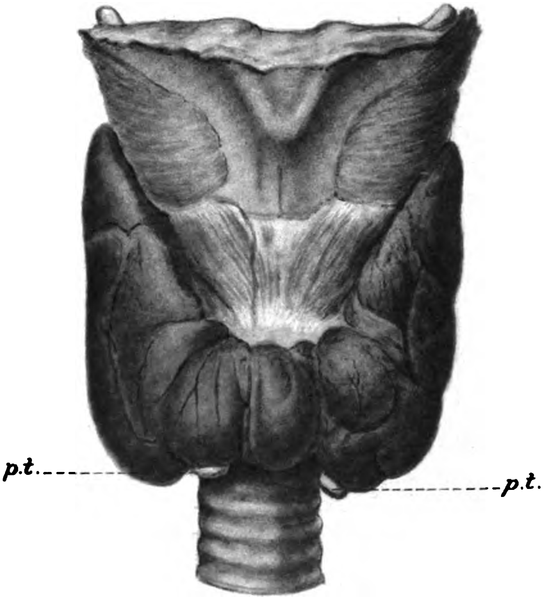
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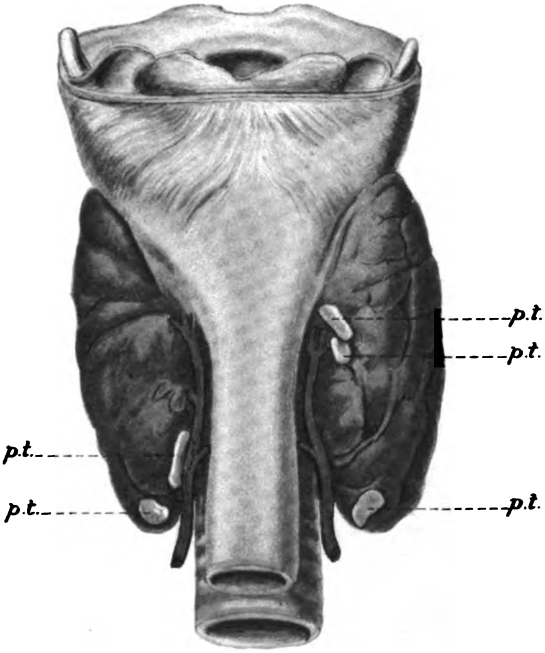
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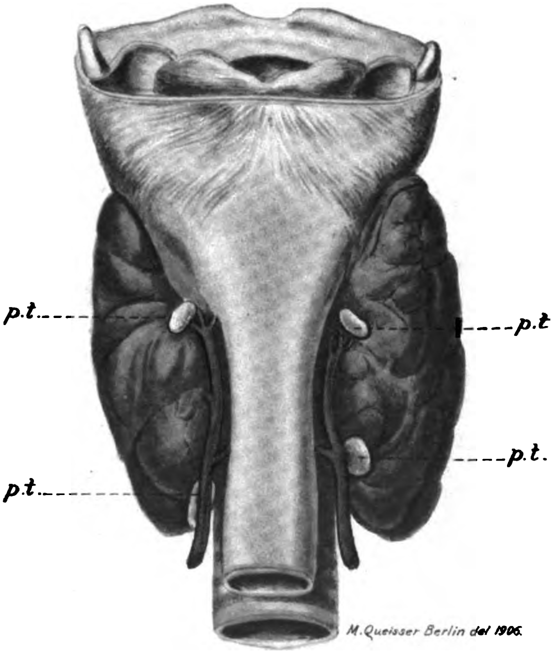
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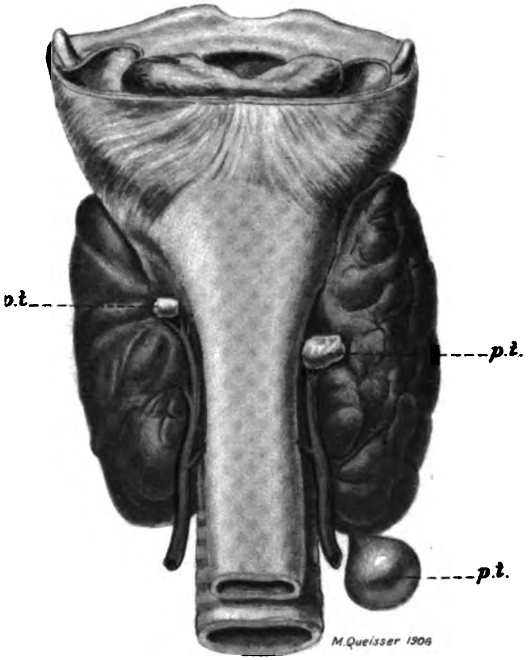

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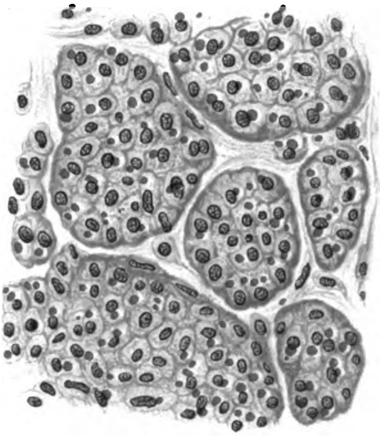

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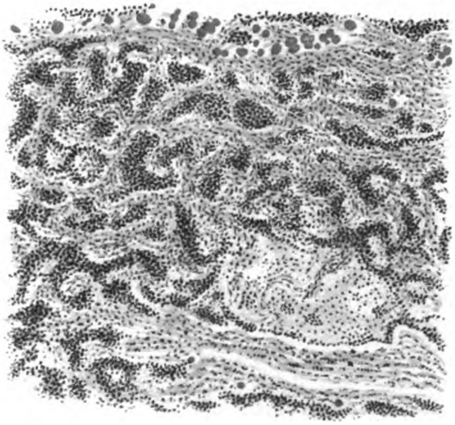

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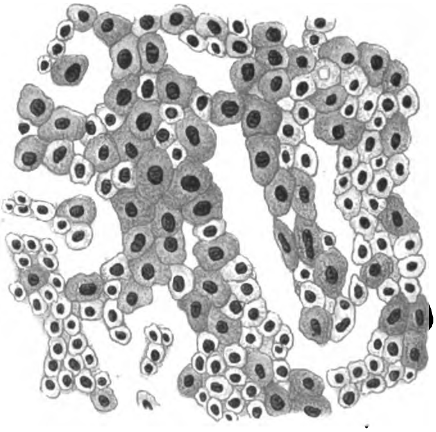

4

THE RESISTANCE OF THE RED BLOOD CORPUSCLES OF THE HORSE TO SALT SOLUTIONS OF DIFFERENT TONICITIES BEFORE AND AFTER REPEATED WITHDRAWALS OF BLOOD.*

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(*Aided by the Rockefeller Institute for Medical Research.*)

In endeavoring to trace the causes leading to certain pathological states in horses used in the production of diphtheria antitoxin and repeatedly bled, one of us found a decided variation from horse to horse in the resistance of the red corpuscles to salt solutions of varying tonicity.‡ When the results were brought together they strongly pointed to repeated bleeding as the cause of lessened resistance, although none of the horses having corpuscles of less than average resistance had been followed from the start. It was also shown that corpuscles of lessened resistance to salt solutions were dissolved more readily in dilutions of their own serum as well as in that of other horses. This fact indicated that the tension of the serum of horses having corpuscles of less than average resistance does not necessarily rise to compensate for this lessened resistance.

The inference that lessened resistance was somehow associated with repeated bleeding was based on the fact that of the seventeen horses examined those most frequently bled possessed blood cells of least resistance. There were, however, among these several exceptions. A mare (No. 70) had corpuscles of relatively low resistance before any blood had been drawn and several horses which had been bled many times still maintained their corpuscles at a level nearly normal. It thus became necessary to analyze this variation

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† During the past year very material assistance in the blood examinations was given by Dr. Paul A. Lewis.

‡ Theobald Smith; The pathological effects of periodical losses of blood. Journal of Medical Research, 1904, n.s. VII., p. 388.

more thoroughly and to determine in how far repeated losses of blood were actually responsible for a low resistance of the blood cells. We have therefore continued the work as outlined in the preceding paper and made large numbers of observations during the past two years, the results of which we shall very briefly discuss in the following pages. Three lines of observation were pursued: 1. About twenty additional horses were examined at different intervals to determine the level of resistance of their corpuscles. 2. A repeatedly bled horse with corpuscles of low resistance was examined from time to time during a long interval of rest to see whether any rise occurred in the resistance of the red cells. 3. Specially selected horses were bled repeatedly and at short intervals.

METHOD. — The procedure described in the preceding paper was followed, with but slight modifications, and the reader is referred to that paper to prevent repetitions. The salt solutions used ranged from .40 to .68 per cent. Great care was exercised in preparing successive solutions uniformly. The salt used for the stock solution was thoroughly ground and dried in a hot air oven at 130° to 140° C. for several hours, and the various dilutions made with the greatest care. We have usually prepared one hundred cubic centimeters of each dilution and used the same a number of times at intervals of a week or more. As a rule a stock solution of one per cent was made from which the various dilutions from .40 to .68 were then prepared. These were kept in old, glass-stoppered, frequently washed bottles, and evaporation avoided as much as possible. Comparative tests were made on the same corpuscles with old and new solutions whenever a new solution was first used. For other details the former paper should be consulted. In the estimation of the percentage tints, difficulties were encountered when the hemolysis was over sixty per cent. To rectify this difficulty two methods were employed. One was to add but one instead of two drops of corpuscles to those tubes in which hemolysis might exceed sixty per cent. The other was to dilute the liquid with two or more volumes of water before estimating the tint.*

A comparison of old and freshly prepared solutions indicated an increase in the tension of the former of from .00 to .02 per cent salt according to age, and relation of volume of fluid to the size of the bottle, that is to say, according to the opportunity for evaporation.

Among those who have studied the resistance of red corpuscles it has been customary to determine two limits, one represented by that strength of salt solution in which the red corpuscles just begin to lose hemoglobin, the other by that strength in which all or nearly all red cells are dissolved. These two limits are known as the minimum and the maximum resistance.

Although this procedure may be very useful in clinical work when very little blood is at the service of the experimenter, it was not considered sufficient for our purposes and we have, as in the earlier work, tested the resistance of the red cells towards intermediate grades of salt solution, using those .02 per cent apart. This gives a much more complete picture of the resistance and enables us to divide the corpuscles into as many classes or groups as there are salt solutions used. For example, in a salt solution of .62 per cent the corpuscles may begin to lose hemoglobin, as is shown by a tint of one-half per cent, in five hours. In a solution of .60 per cent the tint may be one per cent, in a solution of .58 per cent, two per cent. On the basis of these figures we may assume that about one-half per cent of all red corpuscles are destroyed in .62 to .64 per cent solution, one-half per cent in .60 to .62 per cent, and one per cent in .58 to .60 per cent and so on. A still finer division may be made by using solutions only .01 per cent apart, but we have considered this inexpedient owing to the amount of work involved.

VARYING RESISTANCE OF THE RED CORPUSCLES OF HORSES TO SALT SOLUTIONS. — If the hemolysis due to different percentages of salt solutions be plotted by laying out the latter as abscissæ and the percentages of hemolysis as ordinates a curve results. Illustrations of such curves from different horses will be found in Fig 1.

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*The first method introduced an error in that a drop of blood corpuscles has a greater tension than the salt solutions used, but this error is so slight that we have not considered it.

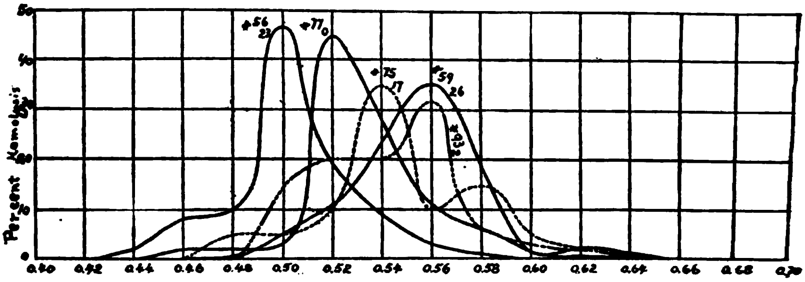

FIG. 1. — Five curves or frequency polygons showing the different groups of red corpuscles as differentiated by salt solutions at the end of five hours. The large number refers to the horse, the smaller number attached to it gives the total number of bleedings of five to six liters each.

The curves plotted from readings taken at the end of five hours teach us that the largest number of red corpuscles have a resistance nearly midway between the maximum and the minimum. Ranged on either side of this mean resistance are diminishing numbers having respectively less and greater resistance than the mean. The curve thus corresponds to a frequency polygon which in normal horses may be classed for the present as a simple unimodal nearly symmetrical curve with range limited in both directions.*

The five selected curves in Fig. 1 illustrate a point already commented on in the preceding paper. The resistance of the corpuscles of horses — leaving aside for the moment the effect of bleeding — is not the same. We may divide horses into classes having corpuscles of maximum, of minimum, and of medium resistance. The extreme difference between those of maximum and those of minimum resistance we have found equivalent to .08 to .10 per cent salt solution, *i.e.*, to nearly one-half of the entire range of resistance of the horse's corpuscles.† Between these extremes all grades of resistance are encountered.* The difference between individual horses is best brought out by comparing observations made on the same day and hence under identical conditions. This simultaneous comparison eliminates possible errors due to slight changes in the salt solutions used. The differences found by a direct comparison of series of tubes are recorded in the following table (I.) in form of an equation. The numbers refer to individual animals, the small figures to the number of bleedings of five to six liters each, and the decimal to the difference found in terms of sodium chloride per cent. Thus $32_{47} = 54_{14} - .04$ means that horse No. 32 (47th bleeding) had corpuscles which were less resistant by about .04 per cent salt than those of No. 54 (14th bleeding); or, expressed in another form ($54_{14} = 32_{47} + .04$), tubes containing corpuscles of No. 54 showed the same amount of hemolysis as those of No. 32, which contained .04 per cent more salt. In order to make the results clearer to the reader, each equation has been entered twice, that is to say once under each animal.

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* Davenport, Statistical Methods, page 19.

† In Fig. 1 the extreme difference is about .06 per cent.

Table I.

$30_{59} = 68_{6} - .01$
$30_{67} = 59_{20} + .04$

$32_{47} = 54_{14} - .04$
$32_{48} = 61_{1} - .065$
$32_{53} = 68_{2} - .06$
$32_{54} = 69_{4} - .08$
$32_{55} = 73_{1} - .05$
$32_{56} = 69_{6} - .065$
$32_{58} = 72_{13} - .045$
$32_{59} = 71_{38} - .065$

$54_{14} = 32_{47} + .04$
$54_{19} = 69_{8} - .06$
$54_{20} = 59_{12} + .01$
$54_{21} = 66_{6} - .04$
$54_{33} = 75_{1} - .02$
$54_{35} = 56_{32} - .05$
$54_{41} = 69_{24} - .04$

$54_{42} = 69_{25} - .03$
$54_{42} = 80_{2} - .01$
$54_{50} = 69_{38} - .05$
$54_{50} = 87_{7} - .01$ to $.04$
$54_{50} = 93_{6} + .015$

$56_{14} = 59_{8} + .06$
$56_{18} = 72_{6} + .02$
$56_{23} = 68_{10}$
$56_{29} = 59_{23} + .06$
$56_{31} = 68_{13} + .025$
$56_{32} = 54_{35} + .05$
$56_{46} = 89_{5} + .01$
$56_{46} = 75_{17} + .05$
$56_{49} = 87_{8} + .01$ to $.04$

$59_{5} = 72_{4} - .05$
$59_{8} = 56_{14} - .06$
$59_{11} = 65_{8} - .07$

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* In the present paper we are not concerned with the rate of hemolysis in any given salt solution. This was discussed and graphically represented in the paper referred to.

TABLE I. — *Continued.*

$59_{12} = 54_{20} - .01$
$59_{13} = 70_{5} - .015$
$59_{20} = 30_{67} - .04$
$59_{22} = 66_{16} - .04$
$59_{23} = 56_{29} - .06$
$59_{25} = 77_{0} - .03$
$59_{26} = 66_{17} - .04$

$61_{1} = 32_{48} + .065$

$65_{3} = 59_{11} + .07$
$65_{7} = 70_{8} + .06$
$65_{12} = 74_{16} + .01$

$66_{6} = 54_{21} + .04$
$66_{16} = 59_{22} + .04$
$66_{17} = 59_{26} + .04$

$68_{2} = 32_{53} + .06$
$68_{6} = 30_{59} + .01$
$68_{10} = 56_{28}$
$68_{18} = 56_{31} - .025$
$68_{18} = 73_{12}$

$69_{3} = 54_{19} + .06$
$69_{4} = 32_{54} + .08$
$69_{6} = 32_{56} + .065$
$69_{9} = 71_{36}$
$69_{14} = 71_{40}$
$69_{24} = 54_{41} + .04$
$69_{25} = 54_{42} + .03$
$69_{25} = 80_{2} + .02$
$69_{33} = 54_{50} + .05$
$69_{33} = 87_{7} + .05$
$69_{33} = 93_{6} + .065$

$70_{5} = 59_{13} + .015$
$70_{8} = 65_{7} - .06$
$70_{9} = 73_{5} - .02$
$70_{14} = 73_{9} - .02$

$71_{36} = 72_{11} - .01$
$71_{36} = 69_{9}$
$71_{38} = 32_{59} + .065$
$71_{40} = 69_{14}$

$72_{4} = 59_{6} + .05$
$72_{6} = 56_{18} - .02$

$72_{11} = 71_{36} + .01$
$72_{13} = 32_{58} + .045$

$73_{1} = 32_{55} + .05$
$73_{5} = 70_{9} + .02$
$73_{9} = 70_{14} + .02$
$73_{12} = 68_{18}$
$73_{12} = 77_{1} - .005$

$74_{16} = 65_{12} - .01$

$75_{1} = 54_{33} + .02$
$75_{9} = 56_{38} - .03$
$75_{17} = 89_{5} - .04$
$75_{17} = 56_{46} - .05$

$76_{2} = 81_{0} + .06$

$77_{0} = 59_{25} + .03$
$77_{1} = 73_{12} + .005$

$78_{1} = 80_{0} - .03$
$78_{1} = 79_{1} + .01$

$79_{1} = 78_{1} - .01$
$79_{1} = 80_{0} - .03$
$79_{6} = 83_{2} - .04$

$80_{0} = 78_{1} + .03$
$80_{0} = 79_{1} + .03$
$80_{2} = 69_{25} - .02$
$80_{2} = 54_{42} + .01$

$81_{0} = 76_{2} - .06$
$81_{3} = 84_{1} - .02$

$82_{10} = 88_{0} + .02$
$82_{10} = 93_{2} + .06$
$82_{13} = 86_{8} + .005$

$83_{2} = 79_{6} + .04$
$83_{8} = 85_{1} + .015$
$83_{8} = 86_{1} + .01$

$84_{1} = 81_{3} + .02$
$84_{8} = 85_{7} + .015$

$85_{1} = 83_{8} - .015$

TABLE I.— *Concluded.*

$85_1 = 86_1 + .005$
$85_7 = 84_8 - .015$
$85_7 = 93_8 + .06$

$86_1 = 83_8 - .01$
$86_1 = 85_1 - .005$
$86_8 = 88_7 + .02$
$86_8 = 82_{18} - .005$

$87_7 = 54_{50} + .02$ to $.04$
$87_7 = 93_6 + .01$ to $.06$
$87_8 = 56_{49} - .01$ to $.04$
$87_7 = 69_{33} - .05$

$88_6 = 93_2 + .04$
$88_6 = 82_{10} - .02$
$88_7 = 86_8 - .02$

$89_5 = 75_{17} + .04$
$89_5 = 56_{46} - .01$

$90_8 = 91_1 - .05$

$91_1 = 90_8 + .05$

$93_2 = 82_{10} - .06$
$93_2 = 88_6 - .04$
$93_8 = 85_7 - .06$
$93_6 = 54_{50} - .015$
$93_6 = 69_{33} - .065$
$93_6 = 87_7 - .01$ to $.06$
$93_9 = 94_1 - .04$
$93_9 = 95_1 - .08$

$94_1 = 93_9 + .04$
$94_4 = 95_1 - .04$

$95_1 = 94_4 + .04$
$95_1 = 93_9 + .08$

A glance at this table shows that horses Nos. 32, 59, 93, 54, 70 and 75 have corpuscles of lower than average resistance. Certain others, such as Nos. 76 and 65, have a resistance above the average.

It must not be assumed, however, that the difference in hemolysis between series of tubes of two horses is necessarily the same from .40 to .70 per cent as might be inferred from the general equation of the table. As a rule, the difference is greater in the lower percentages and grows smaller towards the higher. In some instances it is very uniform throughout, rarely, as in No. 87, it is irregular. The differences given in the table represent most accurately those observed in the middle of the series of salt solutions. The actual differences which cannot be expressed in form of a simple equation are best studied by an examination of the curves in Figs. 1 and 3.

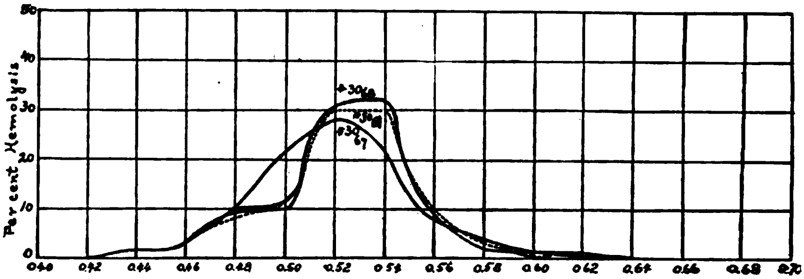

FIG. 2.—Three curves plotted at three successive bleedings one week apart.

THE RELATION OF REPEATED BLEEDING TO CHANGES IN THE RESISTANCE OF RED CORPUSCLES. — In the former paper, as already stated, the preliminary inference was drawn that low resistance was due to repeated bleeding. Additional data on this question have been collected during the past two years. These are too voluminous to present in any detail and we content ourselves with the following brief. synopsis of the experiments:

a. Special cases: No. 32. — In the preceding paper the low resistance of the corpuscles of this horse was described and illustrated by curves. If this condition was due to abstraction of blood, it was reasonable to suppose that a rise in resistance might be anticipated when bleeding was stopped. On October twenty-ninth, 1903, the injection of diphtheria toxin was stopped, but the bleeding continued, at the rate of five and one-half liters every twenty-five days. After March fourth, 1904, only small amounts of blood were drawn for counts and resistance tests. Up to October thirty-first no change in the resistance of the red cells beyond that within the margin of error of the methods employed could be detected.

To determine whether successive withdrawals of blood one week or two weeks apart would have any effect upon the resistance of the red cells, a horse (No. 30) was taken which had been bled sixty-six times since 1900 and whose corpuscles had maintained the average resistance or were perhaps about

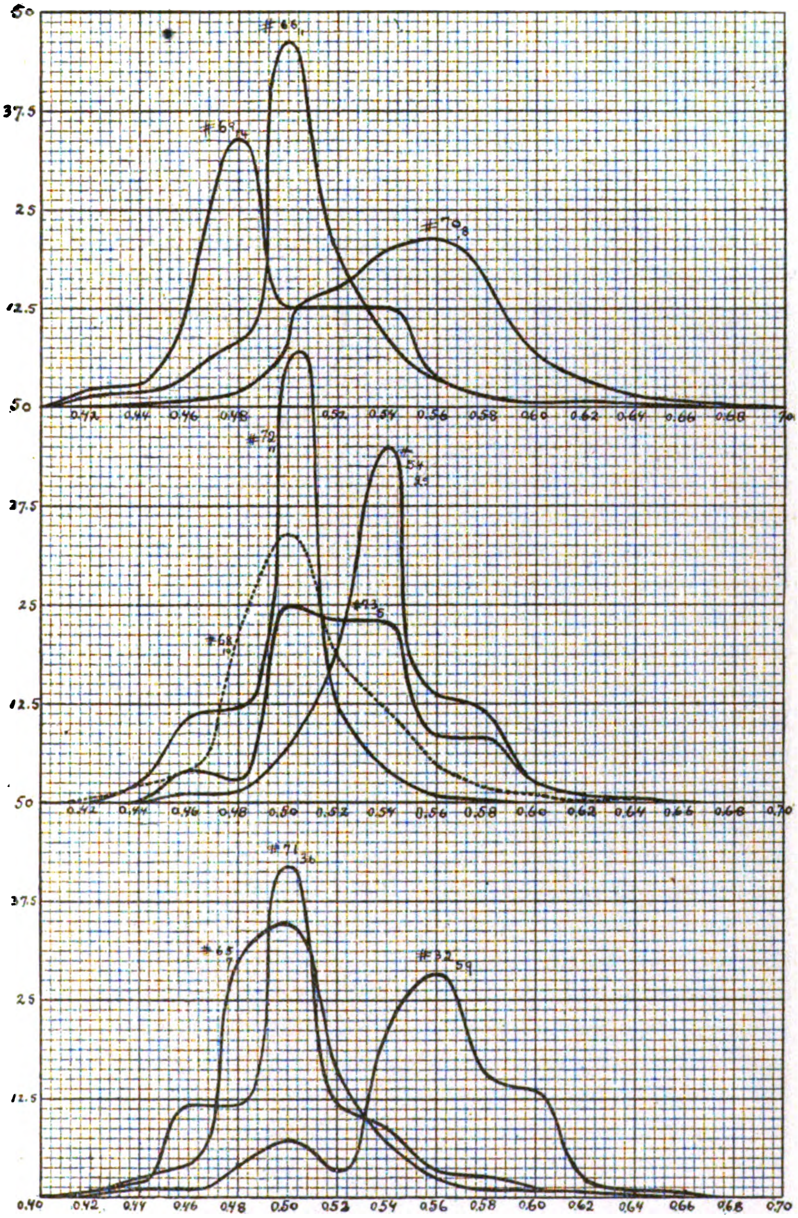

FIG. 3. — Curves from different horses showing the different groups of corpuscles after a twenty to twenty-four-hours' exposure to the salt solutions.

.02 per cent less resistant than the average. After two withdrawals of five and one-half liters within a week, no change in the resistance could be detected one week after the second withdrawal. The three consecutive resistance curves are plotted in Fig. 2. The subsequent history of this horse is given in the article by Dr. P. A. Lewis in this number, page 449.

In another horse (No. 59) whose corpuscles were of a low resistance, the abstraction of five liters of blood (on March 6, 20, and 27) did not make any appreciable impression on the resistance as determined on March 27, April 3, and April 21.

Horse No. 82, weighing eleven hundred pounds, was bled ten times between October fourteenth, 1905, and May third, 1906. Five liters were withdrawn each time. The resistance of the red corpuscles at the tenth withdrawal was about the same as at the beginning.

Horse No. 93 was used as a control. Black mare, eight to nine years old, weighing nine hundred and twenty pounds. Repeatedly bled at intervals of seven to ten days. Received no toxins at any time. The figures in Table I. show that the red cells of this horse had a resistance well below the average from the start.* Between April twenty-seventh and June twenty-fifth, 1906, this horse was bled nine times, five liters being withdrawn each time. A comparison of the protocols of the resistance tests made at the second and the ninth bleeding indicates an average lessened resistance of .015 to .02 per cent salt. This is very slight when we bear in mind that thirty-five liters of blood had been withdrawn between the two tests in a period of about seven weeks. If we allow about seventy pounds of blood for the horse, about eighty pounds, or more than the entire blood in the body at any given time, had been removed in the short period of seven and one-half weeks. There was, however, more or less disturbance in the individual groups of corpuscles as shown in Fig. 4.

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* See also curves in Figs. 1 and 4.

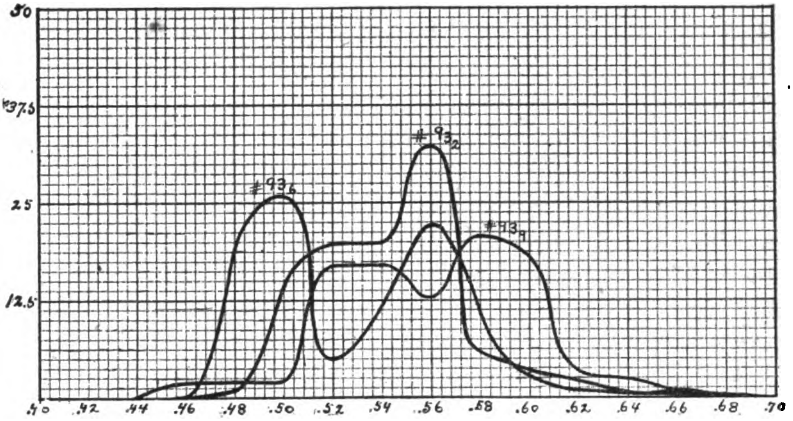

FIG. 4. (See p. 443.)

Horse No. 56, chestnut gelding, about nine years old in 1902. Weight eleven hundred and twenty-seven pounds at that time. This horse was bled fifty times from November, 1902, to July, 1906. The resistance of the red corpuscles, examined first in November, 1903, was found to be normal. Five and one-half liters of blood were drawn every three and one-half to four weeks, with the exception of one period of about six months when the amount drawn was eight liters at a time. A careful comparison of the early records and the latest does not reveal any appreciable fall in the resistance of the red cells.

b. Miscellaneous cases. — Since the preceding paper was written, about twenty additional horses have been studied, of which two (Nos. 82 and 93) have been specially referred to in the preceding pages. All of these have been followed from the beginning. In one (No. 93) the resistance was found at the start nearly as low as in any of the earlier horses. In two others a medium degree of subnormal resistance was found (Nos. 75 and 90).

In none did any marked fall in resistance occur as a result of the usual abstraction of blood, during periods of one to three years. The maximum fall noted has been .02 to .025 per cent salt. Thus in No. 75 this fall was noticed after seventeen bleedings extending over a period of fifteen months. No. 68 showed the same decline in eight bleedings. In other cases the change was not appreciable. Even a change of .02 per cent may fall within the margin of error of the method employed.

These different kinds of data all point to the inference that the extreme differences between certain horses in the resistance of the red corpuscles cannot be accounted for by loss of blood alone. On the contrary, the evidence thus far brought together attributes but a little to loss of blood. The chief phenomenon appears to be individual variation due to still unknown causes just as the capacity to yield diphtheria antitoxin is.*

In no instance have we observed enough change in the resistance of corpuscles to take place during the period of bleeding to permit us to classify an animal of originally normal resistance as one of low resistance. In other words, we have not been able to reproduce those differences by bleeding which we have encountered spontaneously.

THE USE OF SERUM DILUTIONS TO DETERMINE THE RESISTANCE OF RED CORPUSCLES. THE USE OF RED CORPUSCLES TO DETERMINE THE OSMOTIC TENSION OF SERUM.† — In the preceding paper attention was called to the method of comparing the resistance of red corpuscles of horses in dilutions of their own serum as well as in that of other horses, and the conclusion was drawn that this method reveals equally well differences in the resistance of the red cells.

It was recently extended and perfected so that it may take the place of the salt-solution method, provided sufficient control tests are introduced to eliminate the effect of a possible variation in the tension of the serum itself. It is of course inapplicable where the amount of blood obtainable is small.

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* Such individual differences are furthermore expressed in the depth of color of the serum (when free from all hemoglobin), the rapidity with which the clotted blood contracts and with which corpuscles settle down in defibrinated blood. Another more significant variation occurs in the amount of the corpuscular deposit in defibrinated blood. It would be of interest to ascertain whether these variable characters persisting in the individual are transmitted to succeeding generations.

† Hamburger. Osmot Druck, I. S., 359.

In the carrying out of this procedure, the same details should be followed from test to test to ensure uniformity of results. The standard tints prepared were fifty, forty, thirty, twenty-five, ten, five, and two per cent. By taking the standard amount of sedimented corpuscles in three cubic centimeters of water and adding definite amounts of this to serum and water, the above standard tints are easily prepared. It is necessary to use serum because of the color imparted by it. Thus for the above percentages the following dilutions were made with a laked fluid made up in the proportion of two drops of blood corpuscles to three cubic centimeters of water containing a trifle salt to avoid cloudings:

Serum	2	2	2	2	2	2	2	2
Water	0.0	0.0	0.1	0.4	0.55	0.7	0.85	0.94
Laked blood	2.0	1.33	0.9	0.6	0.45	0.3	0.15	0.06
Per cent of tint.....	50	40	30	20	15	10	5	2

It is also desirable to have control tubes of the undiluted and diluted serum, for the serum of horses may differ appreciably in depth of color. Some are pale, watery, others a deep golden yellow. This impresses itself upon the control tints.

As in the tests with salt solutions, small test-tubes were used and definite quantities of serum and distilled water amounting in each case to three cubic centimeters were added to each tube. After shaking the tubes thoroughly one or two drops of sedimented horse corpuscles were added to each tube. This was again shaken up to suspend the corpuscles uniformly. The tubes were kept at room temperature for about five hours, then the tints carefully measured by comparison with a series of controls made with laked blood as described above and the tubes gently shaken again and placed over night in a temperature of 10° to 15° C.

Next morning at the end of twenty to twenty-four hours a final reading was made.

A difficulty arises in the exact estimation of the percentage tint when the hemolysis is forty per cent or above. As this happens frequently with the corpuscles of certain horses it became necessary to meet it. One method was to dilute portions of tubes in which hemolysis was over forty per cent with one or two volumes of water. This was not satisfactory unless serum was used for dilution. A method more economical of time and serum than this is the one referred to above — to add less blood corpuscles — one drop in place of two — to those tubes in which hemolysis may be high and multiplying the resulting tint by two.

The following table illustrates the general method of procedure, and shows at the same time the pronounced differences between the corpuscles of certain horses:

TABLE II.

Serum dilution.			I.	II.	III.	IV.	V.	VI.	VII.
		Serum of	No. 93.	No. 95.	No. 93.	No. 94.	No. 94.	No. 95.	No. 93.
Serum + water, cc.	Per cent serum.	Corpuscles of	No. 93.	No. 93.	No. 94.	No. 94.	No. 93.	No. 95.	No. 95.
1.7 + 1.3	56⅔	Per cent hemolysis after 24 hours.	100	80	80	80	100	10	30
1.8 + 1.2	60		90	60	50	40	85	6	12
1.9 + 1.1	63⅓		70	14	15	12	60	Trace.	5
2.0 + 1.0	66⅔		30	5	5	3-4	30	0	Trace.
2.1 + 0.9	70		10	1-2	Trace.	Trace.	S	0	0
2.2 + 0.8	73⅓		3	?	0	0	3	0	0
3.0 + 0.0			0	0	0	0	0	0	0

From this table we may obtain information about the resistance of the red corpuscles as well as the osmotic tension of the serum.

Comparing columns I. and II., which show the percentage hemolysis of corpuscles of 93 in serum of 93 and 95, we find that the osmotic tension of serum 95 is four to five per cent serum higher than 93. Comparing columns VI. and VII. we see again that the osmotic tension of serum 95 is about four per cent serum higher than 93. Comparing columns I. and V., and columns III. and IV. we learn that the osmotic tension of the serum of 93 and 94 is practically the same. It will be seen that this determination is made by adding corpuscles of the same horse to the sera of different horses.

Turning now to the resistance of the corpuscles, we may determine this from the table by comparing the hemolysis of the corpuscles of different horses placed in the same serum. Thus, comparing columns I. and III. and columns IV. and V. we learn that the difference in resistance between the corpuscles of 93 and 94 is four to four and five-tenths per cent serum. Knowing that the tension of serum 93 and 94 is the same, we conclude that corpuscles 93 are less resistant than those of 94 by four to four and five-tenths per cent serum.

Comparing the resistance of corpuscles of Nos. 93 and 95 (columns II. and VI., and I. and VII.), we find that the difference in the first case is seven per cent serum, in the second ten per cent in favor of No. 95. This slight discrepancy cannot at present be explained. It seems to indicate that after eliminating the difference in serum tension the corpuscles of 93 are laked more easily in their own serum than in that of 95.*

Finally, comparing columns III. and VII. the difference between corpuscles of 94 and 95 is about four per cent serum. Thus we obtain the independent equations:

(1) Corpuscles of 93_9 = corpuscles of $94_1 - 4$ to 4.5 per cent serum.
(2) " " 95_1 = " " $93_9 + \frac{1}{2}(10 + 7) = 8.5$ per cent serum.
(3) " " 95_1 = " " $94_1 + 4$ per cent serum.

From (1) and (3) we also get:

(4) $95_1 = 93_9 + 8$ to 8.5 per cent, which is identical with (2).

Comparing the results obtained with salt solutions at the same time we have the following close agreement:

$93_9 = 94_1 - .04$ per cent salt solution.
$93_9 = 95_1 - .08$ " " " "
$95_1 = 94_1 + .04$ " " " "

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* This has been observed in another case, though the difference amounted to but one per cent serum.

We note that the per cent of serum is nearly the same as the hundredths per cent salt in these equations. This is due to the fact that the osmotic tension of serum is equivalent to about 0.9 per cent salt solution. This figure is obtained by comparing the per cent hemolysis in the salt solutions and in the serum dilutions. Thus if the laking effect of sixty per cent serum is the same as that of 0.53 per cent salt solution, the tension of undiluted serum would be $\frac{.53 \times 100}{60} = .88$ per cent salt solution.

In the same manner the following data concerning serum tension and corpuscle resistance were determined. These it will be noted agree very closely with the data obtained with simple salt solutions.

Table III.

Comparison of the Serum Tension of Two Horses in Per Cent of Serum.	Resistance of the Corpuscles of Two Horses Compared. The Difference Expressed in Per Cent of Serum.
$56_{46} = 75_{17} + 5\%$	$56_{46} = 75_{17} + 6\%$
$56_{46} = 89_{5}$	$56_{46} = 89_{5}$
$59_{8} = 63_{2}$	$69_{6} = 32_{56} + 6\frac{2}{3}\%$
$69_{6} = 32_{56}$	$69_{33} = 54_{60} + 4$ to 5%
$69_{33} = 54_{60}$	$69_{33} = 87_{7} + 4$ to 5%
$69_{33} = 87_{7} - 4$ to 5%	$75_{17} = 89_{5} - 5\%$
$79_{6} = 83_{2} - 3\frac{1}{3}\%$	$79_{6} = 83_{2} - 4\%$
$80_{1} = 78 + 1\%$	$82_{10} = 86_{7} + 1\frac{2}{3}\%$
$82_{10} = 86_{7}$	$82_{10} = 88_{6} + 4\%$
$85_{7} = 84_{8} + 1\frac{2}{3}\%$	$85_{7} = 84_{8} - 2$ to 3%
$86_{7} = 93_{6} + 1\frac{2}{3}\%$	$86_{7} = 88_{6} - 3\frac{1}{3}\%$
$88_{6} = 86_{7} + 1\frac{2}{3}\%$	$93_{9} = 94_{1} - 4$ to 4.5%
$93_{9} = 94_{1}$	$95_{1} = 94_{1} + 4.5\%$
$93_{9} = 95_{1} - 4\%$	$95_{1} = 93_{9} + 8\%$

As stated in the preceding paper, serum tension and resistance of red cells are not parallel to each other. In the table we find horses having corpuscles of low resistance whose serum tension is low (No. 75), normal (Nos. 32, 54), and high (No. 87).

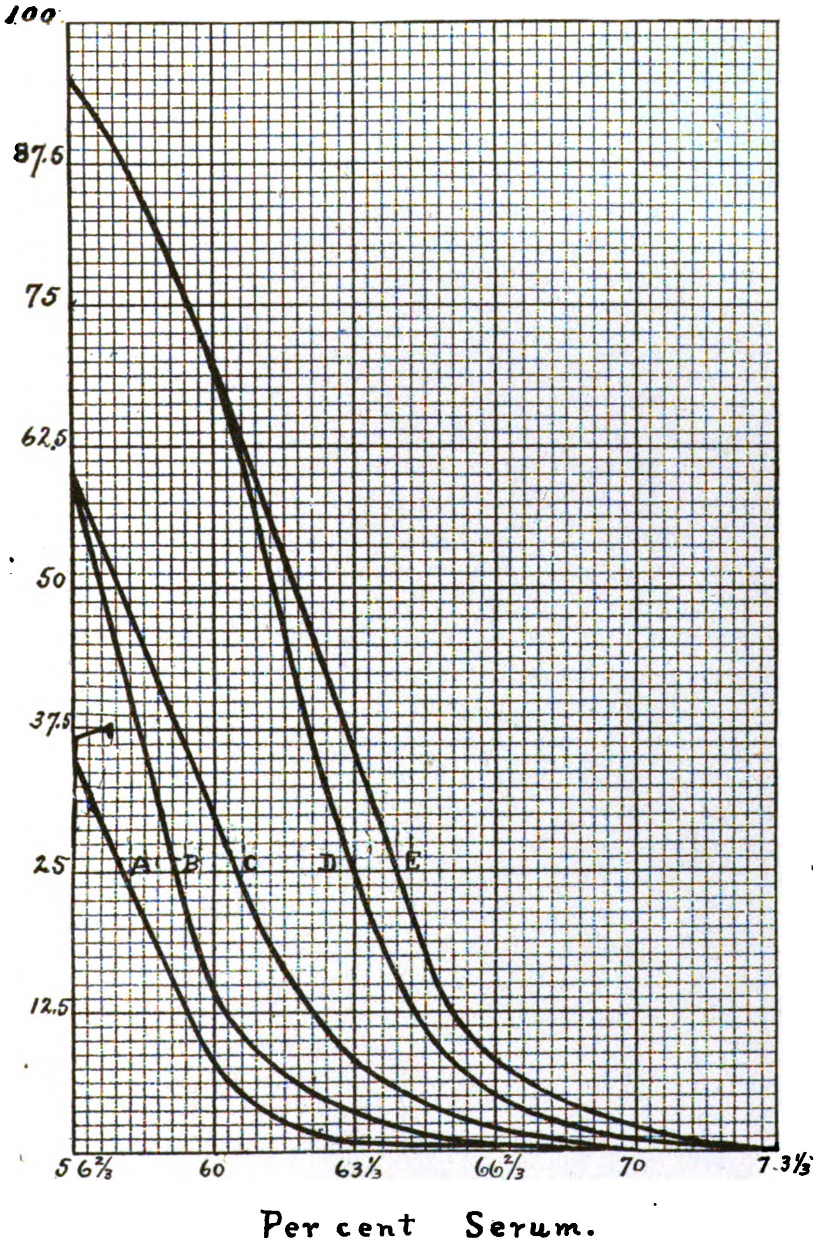

FIG. 5.—Curves constructed by plotting the total hemolysis on coördinates over the serum dilutions on the axis of abscissæ. The serum used in all cases is that of horse No. 86. The corpuscles tested are from No. 82 (A), 86 (B), 88 (C), 93 (D, E). The tests recorded by B, C, D were made twenty-four days later than those of A and E. It will be noticed that corpuscles of No. 82 are the most resistant, those of No. 93 the least.

Our records are not complete enough to enable us to state whether this serum tension is, like the resistance of the red cells, a fairly constant individual factor or one varying from time to time. This would most probably be determined with greater accuracy by freezing-point determinations which eliminate the chance (which, however, is very slight indeed) of variations in the resistance of corpuscles of the same horse used to test the serum tension from time to time.

ISOLYSINS. — In the use of serum dilutions to determine both the resistance of red corpuscles and the osmotic tension of the serum, it was shown that by placing the corpuscles of the same horse into different sera, and the corpuscles of different horses into the same serum these objects could be attained and the errors resulting from different tonicity of sera eliminated. There is, however, one source of error recently encountered to which attention should be called. It is the existence of isolysins in a small number of horses, perhaps ten per cent. These had been sought for from the start, but none found, hence the statement in the former paper that isolysins were not encountered.

Two horses having in their serum lysins towards the corpuscles of other horses have been under observation. Though both these animals are producing antitoxin, it is assumed that the lysin is not the result of any toxin treatment. Lysis of red corpuscles of other horses up to fifteen per cent results in undiluted serum and the increased hemolysis over and above that due to dilution of the serum is evident throughout the series. Very recent tests indicate that it is heat-labile and disappears when the serum is heated at 55° C. for one-half hour. These isolysins will be studied more in detail and form the subject of a later communication. One object in referring to them here is to warn any future observers of the existence of these bodies and point out that they interfere with resistance tests in diluted serum.

GENERAL CONSIDERATIONS AND SUMMARY.

About ten per cent of the horses which have come under observation have red corpuscles of a resistance so low as compared with the other ninety per cent that even crude methods could have detected it. The use of accurate methods carried out uniformly from month to month showed little if any change or fluctuation in this low resistance over a period of one to three years. The same results were observed when dilutions of horse serum were employed in place of salt solutions. As shown in Table III. the osmotic tension of the serum from various horses was the same with few exceptions, whereas the resistance of the red corpuscles was subject to considerable fluctuations, from case to case.

The influence of repeated bleedings has been shown to be relatively slight. In a few horses there was noticed a lowering of resistance equivalent to .02 to .025 per cent salt solution.

It is highly probable that with more severe losses of blood some impression can be made upon the inner form of the frequency polygon, which represents graphically the different groups of corpuscles. The only horse which we have subjected to any severe bleeding was No. 93. This animal has already been referred to. A plotting of three blood examinations is shown in Fig. 4. The earliest curve (93_2) approximates the unimodal curve. The curves obtained from corpuscles at the sixth and ninth bleedings depart markedly from the normal and indicate a decided rearrangement of the various groups of corpuscles, resulting in bimodal curves. It would appear as if the severe drain brought into the circulation more corpuscles of a given group than occur normally in the blood. We may then assume, pending other trials of severe blood-letting within a short space of time, that the monthly losses of one-seventh of the total blood is well borne and does not appreciably change the form of the frequency polygon or curve of resistance as we have plotted it, but that severe losses may modify the curve without affecting appreciably the maximum and the minimum resistance.

Turning to some physiological aspects of the variation in resistance, we have to consider its relation to sex and age of the individual, and to age and size of the red cells themselves. Before doing this it may be well to enquire what the normal resistance to salt solutions may be considered to be. Inasmuch as we have variates ranging themselves on either side of the mean, this mean may be called normal for purposes of standardization and comparison of results. A tabulation of the various results obtained leads us to establish two points for the horse which can be used for comparison. (1), The total hemolysis in .50 per cent salt solution, and (2), the salt solution corresponding to fifty per cent hemolysis. For (1) the mean or normal is thirty to forty per cent hemolysis. For (2) the mean or normal is .49 per cent salt solution. Or, stated in round numbers, in the average horse .50 per cent salt causes a total of fifty per cent hemolysis, and hence fifty per cent hemolysis is caused by .50 per cent salt solution.

The following table exemplifies the rule and readily reveals the horses with corpuscles of low resistance:

TABLE IV.

Number of Horse.	Per Cent Salt Solution Corresponding to 50% Hemolysis.	Per Cent Hemolysis Corresponding to .50% Salt Solution.
3061	.505	60
3259	.546	85
5425	.529	90
5623	.492	33
5916	.554	95
699	.491	33
708	.535	80
7517	.537	90
84	.493	35
851	.492	38
911	.492	38
939	.552	92
941	.51	64
951	.473	25

The relation of sex to resistance of red corpuscles is quite striking. Of thirty-eight horses examined during the past three years, twenty-one were males and seventeen females. Of the six with corpuscles of low resistance all were females.* In age all the horses examined ranged from eight to possibly twenty years. The same differences in age are found among those with corpuscles of low resistance.

This low resistance may be a phenomenon of early life which in some individuals does not change as the animal becomes adult. We have not been able to examine young horses to clear up this point. If it should prove to be characteristic of the young, its persistence in later life may be a racial character evolved by breeding. It is not peculiar to either well-bred or ordinary breeds, for we have been able to examine both kinds and we do not find it belonging to one or the other class.

Turning now to the red corpuscles themselves we may ask whether there is any relation between resistance to salt solutions and age. This is a purely physiological question, but it seems desirable to point out certain inferences which may be drawn from our data on this point. Hamburger† assumes that there is such relationship, but does not define it. Under this assumption there are two possible theories. First, the youngest corpuscles may be least resistant; second, the oldest corpuscles may be least resistant.

Assuming that the youngest corpuscles are the most resistant and that the oldest are the least resistant and the ones destroyed, we would have for our polygon not the one we have figured but one rectangular in outline. If the youngest corpuscles are the least resistant to salt solutions and oldest corpuscles the most resistant, but still the ones to be destroyed, the polygon would also be rectangular. If the youngest corpuscles were the most or the least resistant and if the destruction were haphazard and liable to invade any group of cells the curve would be irregular and dependent upon accidents.

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* It should also be stated here that the two horses whose serum contained isolysins were females. One of them had corpuscles of normal resistance, the corpuscles of the other were below normal.

† Osmot Druck, I., p. 363.

Neither of these hypotheses accords with the facts. The theory most in harmony with them disavows any relationship between age of corpuscles and resistance to salt solutions, and assumes that the different groups are produced and destroyed independently of their resistance to salt solutions. The difference in the various groups may perhaps be due to their different origin, some coming from the marrow of the ribs, others from that of the head of the femur, still others from the shaft of the same bone and so on. This theory might account for the abnormal curves in No. 93 (see Fig. 4). Places in the osseous system of this horse hitherto called upon but slightly may have furnished large numbers to meet the severe drain.

The possible relation between diameter of corpuscles and their relative resistance to salt solutions was taken into consideration. A large number of measurements were made, but the results did not show any close correspondence between size and resistance. Nevertheless we do not wish to convey the impression that our work is in any way final. Here, also, more repeated and larger bleedings may be necessary to bring about such exaggerated conditions as are suitable for a further study of this problem.

Turning finally to the pathological significance, the question naturally arises, is the animal with corpuscles of low resistance more prone to certain diseases than the one with corpuscles of normal resistance? The data collected during the years of observation indicate that the former are no more predisposed than others to the ill effects of toxin injections and repeated bleeding. This is brought out in the following paper by Dr. P. A. Lewis. Another disease to which the horse is subject is azoturia or hemoglobinuria. This is characterized by the presence of hemoglobin in the urine. Large draught horses are most susceptible and the disease occurs chiefly in winter.

Among our animals there were five which had wholly or partially recovered from azoturia. Of these only one (No.

32) had corpuscles of low resistance. This showing is hardly sufficient to associate azoturia with such corpuscles.

The relation of low resistance to any increased susceptibility to specific hemolysins has not been investigated.

Our results cannot be generalized to other species, but these must be studied in the same laborious way to determine to what extent they are influenced by losses of blood and how far individual, hereditary or developmental characters enter to cause original differences in the resistance.

Attention should be drawn, however, to the uncertainty now surrounding the resistance tests of human corpuscles hitherto recorded. Two facts stand out quite preëminently in our results, individual variation and marked stability of this variation. In the human subject statements of lowered resistance cannot be fully accepted until individual and racial variations in health have been studied. If such variations are found to occur then the resistance formula for any diseased condition must be preceded by the normal formula for that individual, for it may occur that, as in the horse, the individual, normal variation is greater than any attributable to disease.

On the whole, it may be said that the study of the resistance of red corpuscles to different osmotic tensions does not appear to have much pathological significance if such extreme differences as those we have found in horses are compatible with health and repeated losses of blood. The subject nevertheless merits more attention on the part of clinical medicine. Statistical observations among human beings living in both temperate and tropical climates are especially to be desired. It is in the tropics where the osmotic tension of the serum may be subjected to far greater fluctuations than in the temperate zones. There, also, a high degree of immunity toward intraglobular parasites exists.

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HEMORRHAGIC HEPATITIS IN ANTITOXIN HORSES.*

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(*Aided by the Rockefeller Institute for Medical Research.*)

About six years ago the attention of the director of these laboratories was called to unusual developments in certain horses used to produce diphtheria antitoxin. Some horses after years of successful use would die suddenly, post-mortem examination revealing, as the immediate cause of death, extensive hemorrhage into the peritoneal cavity from a ruptured liver. Because of its practical importance to those engaged in the production of antitoxins and its possible theoretical significance, this subject was thought worthy of the most careful investigation in its diverse aspects. The earliest work on the subject seemed to indicate that pathological changes in the red blood corpuscles might be the basis of the disease. Experiments primarily conducted with a view to deciding whether or not this were true have been carried to completion and the results have been published.† They show that the erythrocytes are but little altered in their resistance to hemolysis in hypotonic sodium chlorid solutions by immunization and repeated bleeding, and that the osmotic tension of the serum is not essentially changed.

The work which forms the basis for this paper consisted in a study of the records of the treatment, and a careful gross and microscopic post-mortem examination of such horses as died or were killed. In some instances the usual routine treatment for the production of antitoxin has been modified by an increase in the frequency of bleeding. One horse not treated with diphtheria toxin and one treated for a short time only have been bled repeatedly, killed by shooting and studied anatomically.

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* Received for publication Nov. 4, 1906.

† See preceding paper. — *Ed.*

This work shows that prolonged treatment of the horse with diphtheria toxin, bleeding repeatedly during the same period, causes amyloid degeneration of the liver and, less often, of the spleen. After amyloid degeneration of the liver has developed, increasing the frequency of the bleeding, while keeping the quantity of blood drawn each time, a constant, brings about hemorrhage into the organ, which may rupture through the capsule and terminate fatally. In its earliest stage the hemorrhagic lesion is an infarct produced by the obstruction to the circulation through the sinusoids of the peripheral portions of the liver lobule.

The preliminary work on this condition was done by Dr. Theobald Smith and he has been actively interested in its completion. I thank him sincerely for the opportunity he has afforded me for an interesting and profitable study.

While this disease of the horse is essentially an artificial or experimental one, taking its origin in the new service into which the animal has been pressed in recent years, it is none the less, from the point of view of morbid anatomy, closely allied to if not identical with, conditions that have long been known to veterinarians. The standard text-books on the pathological anatomy of domesticated animals have short chapters devoted to amyloid liver and rupture of the liver in the horse, and the relation of the one condition to the other is usually commented on. Under these titles Friedberger and Frohner in the appendix to the latest edition of their text-book give a complete index to the literature of these subjects referring to the diseases arising under natural conditions. Their list includes seventy titles.

It is probable that these diseases of the horse are much more common in Europe than in this country. Nevertheless the possibility of encountering a naturally diseased animal would have to be borne in mind constantly while conducting experiments along these lines and any too striking result may have to be criticised with such a coincidence in mind. Whether the very definite series of anatomical changes which our cases present is constant in the apparently similar cases arising under natural conditions is perhaps doubtful.

In a discussion on amyloid degeneration at the meeting of the Deutsche Pathologische Gesellschaft in 1904 this condition was considered in part, and in its present relationship. In this discussion Zenoni was credited with observing amyloid in the livers of antitoxin horses and Kraus considered that the observation was especially important, as it might afford an opportunity for comparing amyloid degeneration artificially produced with that arising in the same species of animal under natural conditions.

At the recent meeting of the American Association of Pathologists and Bacteriologists (Baltimore, 1906), Pearce and Pease gave a preliminary report of pathological lesions in antitoxin horses. Their paper has not to our knowledge been published. A communication they kindly sent us states that amyloid degeneration has been present in but two of their cases and that thrombosis of the veins has been an important feature in most of them.*

Our material comprises eleven horses. The accompanying table shows the chief facts concerning the horses, their treatment and the result.

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* About three years ago the vice-director of the Serum Institute in Bern told Dr. Smith that some of their horses had died of hemorrhage from the liver. Nothing has to our knowledge been published from that laboratory.

TABLE I.

Summary of horses, treatment and results.

Number of Horse.	Sex.	Age.	Time Under Treatment.	Cause of Death.	Treatment.	Found at Post-mortem Examination.
III.	Male.	12 years.	5 years.	Shot.	Diphtheria toxin. Maximum 500 cc. periodically. Bled 54 times. Interval — 53d to 54th, 1 week.	Extreme amyloid degeneration of liver. Sub-capsular hemorrhage several inches in diameter. Microscopic hemorrhages into centers of liver lobules. Dilatation and rupture of central veins.
XVII. . .	Female.	. . . . . .	4 years.	Rupture of liver hemorrhage.	Diphtheria toxin. Maximum 500 cc. periodically. Bled 47 times. No increase in frequency. Died in morning after last bleeding.	Extreme amyloid degeneration of liver. Large recent hemorrhages into liver. Rupture of liver. Hemorrhage into peritoneal cavity. Old encysted hemorrhages of liver.
XXX. . .	Male.	12 + years.	5½ years.	Rupture of liver hemorrhage.	Diphtheria toxin. Maximum 500 cc. periodically. Bled 71 times. Intervals — 67th to 68th, 1 week 68th to 69th, 1 week 69th to 70th, 1 week 70th to 71st, 1 week } Died one month after last bleeding.	Extreme amyloid degeneration of liver. Recent large hemorrhages. Rupture of liver. Hemorrhage into peritoneal cavity. Old partly organized hemorrhages of liver. Regeneration of liver parenchyma.
XXXII. .	Female.	11 years.	4½ years.	Shot.	Diphtheria toxin. Maximum 600 cc. Bled 59 times. Toxin stopped for one year. Bleeding stopped for eight months. Then bled twice with interval of one week. Killed one week later.	No amyloid degeneration. No hemorrhage.
LIX. . .	Female.	12 years.	2½ years.	Shot.	Diphtheria toxin. Maximum 500 cc. Bled 25 times. Intervals — 22d to 23d, 2 weeks. 23d to 24th, 1 week. 24th to 25th, 1 week.	Trace of amyloid degeneration of liver. Well marked amyloid degeneration of spleen. Acute hemorrhagic nephritis.

LXXI. . .	Male.	12 + years.	6¼ years.	Shot.	Typhoid toxin. Five years. 20–100 cc. Diphtheria toxin. 1½ years. Maximum 500 cc. Bled 41 times. No increase in frequency of bleeding.	Well marked amyloid degeneration of liver. Extreme amyloid degeneration of spleen.
XI.	Male.	9 years.	2 years.	Septicemia secondary to strangulated hernia.	Diphtheria toxin. Maximum 700 cc. Bled 13 times. No increase in frequency of bleeding.	No amyloid degeneration. No hemorrhage.
XXIII. . .	Male.	15 years.	3½ years.	Shot.	Diphtheria toxin. Maximum 500 cc. Bled 48 times. No increase in frequency of bleeding.	No amyloid degeneration. No hemorrhage.
LVI. . . .	Male.	12 years.	3½ years.	Shot.	Diphtheria toxin. Maximum 500 cc. Bled 52 times. Last five bleedings at intervals of one week.	No amyloid degeneration. No hemorrhage.
LXXXII. .	Female.	14 years.	¾ year.	Shot.	Short treatment with diphtheria toxin. Rested. Bled 11 times. Frequency increased at last.	No amyloid degeneration. No hemorrhage.
XCIII. . .	Female.	10 years.	3 months.	Shot.	No toxin. Bled 10 times at intervals of about one week.	No amyloid degeneration. No hemorrhage.

It is my purpose to consider these facts from several points of view which are more or less distinct from each other.

I. The experimental production of amyloid.
II. The effect of bleeding on normal horses and on horses with amyloid degeneration of the liver.
III. The pathological anatomy of horses treated with diphtheria toxin and subjected to repeated bleedings.

I. *The experimental production of amyloid.*

Under this heading we have nine horses, ranging from nine to fifteen years in age, that have been injected with diphtheria toxin over considerable periods of time and bled at intervals. The horses after purchase are immunized by increasing doses of toxin, as rapidly as is possible without producing pronounced failure in health, until the antitoxic value of the serum reaches at least two hundred units. At the end of two months the horse is usually receiving about two hundred cubic centimeters of toxin at each injection. For the remaining period of the treatment the horse receives two or three toxin injections between consecutive bleedings which are about twenty-five days apart. The horse is not bled until ten days after an injection of toxin. After the antitoxin becomes strong enough for use the dosage of toxin is still further gradually increased. The maximum dosage over long periods of time is about five hundred cubic centimeters of a toxin whose minimum fatal dose is about .005 to .006 of a cubic centimeter. At each bleeding about five liters of blood per thousand pounds of weight are drawn. Under these conditions five-ninths of the animals autopsied have suffered with well marked amyloid degeneration of the liver or spleen or of both organs. The shortest period of treatment giving a positive result is two and a half years. Beyond this time the longer a horse is treated the more certain he is to suffer this degeneration. At three years and a half two of the horses were negative. Of four horses treated, four years and over, three showed amyloid.

We have attempted to determine what part one factor in the treatment of these horses, that is the repeated bleeding, has in the development of the degeneration. The cases of horses 93 and 82 show that severe repeated bleedings at weekly intervals for ten weeks do not cause amyloid degeneration. The case of horse 56 shows that even when preceded by a three and one-half year period of mixed treatment five bleedings at intervals of one week do not develop the degeneration. So far as it goes the evidence is that the crude toxin injected is the factor of prime importance in the production of amyloid.

Microscopic examination shows that the distribution of the amyloid produced by this method is always the same with relation to the tissue and the individual cell. In all cases it appears in the liver, although in two it is more abundant in the spleen. These two are the only cases in which the spleen is affected. The horse which showed most amyloid in the spleen was treated for five years with typhoid toxin and for one year with diphtheria toxin. Whether this variation from the usual treatment accounts for the localization in the spleen rather than the liver seems doubtful in view of the case of horse No. 59 which under the influence of the diphtheria toxin developed more splenic than hepatic degeneration.

II. *The effect of repeated bleeding.*

While the amyloid degeneration cannot be positively referred to any one factor in the treatment our results throw more light on the cause of the hemorrhage. As may be seen by reference to the table there is marked hemorrhage into the liver in three of the horses — two of them dying by rupture of the organ and intraperitoneal hemorrhage. All of these horses show amyloid degeneration of extreme grade in those portions of the liver which are hemorrhagic. In all there was amyloid throughout the liver and hemorrhage into parts. In the case of the two horses in which hemorrhage did not occur the amyloid degeneration is less severe. In one of them it is inconsiderable. In the horses showing amyloid throughout the organ and hemorrhage into portions of it, the non-hemorrhagic areas show less amyloid than the hemorrhagic ones. None of the animals in our series had hemorrhage without amyloid degeneration. From these facts we conclude that amyloid degeneration in the liver of the horse is a serious predisposing factor toward hemorrhage into, and rupture of the organ. While we recognize the possibility that the same final result might be reached by another mechanism entirely, in our own cases the only precursor of the liver hemorrhages that can be demonstrated anatomically is amyloid degeneration.

Those who have noted the association of liver rupture with amyloid degeneration have ascribed it to the greater brittleness either of the liver tissue as a whole or of the affected vessel walls. The friability of the liver tissue was noticeable in our cases, but there seems to be another factor which may influence the hemorrhage. The hemorrhage, in fact, occurs only in those cases and in those parts of the liver where the amyloid degeneration is most extreme, and where the circulation through the sinusoids is seriously impeded or completely obstructed. This obstruction is always peripheral in the lobule and where it is practically complete the result is to throw the full force of any regurgitant wave from the right heart into the center of the lobule. The normal liver tissue of the horse is not very much stronger than that of smaller animals, and the heart is of course much more powerful. We have fulfilled in these cases the conditions for a hemorrhagic infarction of the liver as laid down by Chiari for human cases. In his cases the obstruction to the portal and peripheral lobular circulation was due to tumor metastis. That this is the nature of the hemorrhagic lesions in one of our cases is shown by the histological examination of the liver of horse No. 3. Sections in several instances show diffuse infiltrating hemorrhages into the center of the liver lobules with extensive amyloid degeneration of the peripheral half. In one instance the wall of the central vein is ruptured, hyaline fragments of it remaining. In other instances the central vein is irregularly dilated to twice its normal size, the wall is eccentrically thinned, but as yet unbroken. The central portions of the cords of cells are compressed. Amyloid degeneration of the peripheral portions of the lobule is extreme. Our other cases throw no light on the mechanism of the hemorrhage. The lesions are large. The tissue relations are greatly altered. The large masses of blood clot are bordered by liver tissue in many stages of necrosis, all of which may be ascribed to secondary tearing and pressure. Our cases do not show necrosis or thrombi in situations that would lead one to suspect that the hemorrhage was secondary to them.

We think that the hemorrhages in our cases resulted from the extension and fusion of multiple lobular hemorrhagic infarctions caused by amyloid degeneration so extreme as to obstruct the sinusoidal circulation at the periphery of the lobule. The process once started the extension is favored by the friability of the amyloid liver.

It is probable from our experience that disturbances of the circulation or perhaps undemonstrable changes in the composition of the blood incident to bleeding may determine the infarction and hemorrhage when the anatomical foundation is laid. The first horse lost here died early the next day after being bled. There had been a rise in temperature to one hundred and three degrees immediately after bleeding. There had also been a rise in temperature two weeks before at the time of the last toxin injection. A consideration of these facts led to the following experiments which have been carried out at intervals as the horses became available.

Horse XXX. — Treated five and one-half years. No toxin during the last six weeks. Drew three and one-half liters of blood four times at weekly intervals. One month after the fourth bleeding the temperature rose to one hundred degrees. The animal was sick. Four days later three liters of blood were drawn. Died in the night. Amyloid — hemorrhage — rupture of liver. Up to the time the frequent terminal bleedings were commenced the animal was in apparent good health, and there seems no reason to doubt that the frequent large bleedings were responsible for the acute development of the hemorrhagic lesions in the liver and that the last bleeding hastened the death of the horse in the same way.

Horse III. — Treated five years. Bled fifty-four times. During the period there was no marked rise in temperature. After the usual interval was bled July 16. July 23 bled again. No symptoms. July 27, shot through the brain. Extreme amyloid degeneration of liver. Hemorrhage several inches in diameter beneath the capsule. Microscopic examination shows many hemorrhages into centers of lobules. Hemorrhages show no evidence of organization.

As a control on these horses that have shown extensive hemorrhages having an apparent relation to the bleeding there is one horse (No. 71) showing well marked amyloid disease but no hemorrhage. This animal was never bled oftener than once in twenty-five days.

Three cases, horses Nos. 22, 56 and 59, show that the long continued routine treatment with toxin and bleeding does not predispose to hemorrhage when the frequency of bleeding is increased, unless amyloid degeneration is produced. That is, the treated horse in which the liver remains normal anatomically behaves under frequent bleedings as does a normal animal.

In two experiments an attempt was made to determine the share of continued bleeding in the production of the whole disease.

Horse LXXXII. was treated and bled four times from August, 1905, to January, 1906. Toxin was then stopped. He was bled January 25, March 8, March 26, April 13, April 27, and then at intervals of five to nine days until the total number of bleedings was eleven. Shot. No lesion.

Horse XCIII.—Never had toxin. From April 27, 1906, to July 2, 1906, he was bled ten times, the intervals being about one week. Five and one-half liters of blood were drawn each time. July 3, shot. No lesion.

These experiments strengthen the view that the frequent bleedings only act to produce hemorrhage when preceded by amyloid degeneration. They of course throw but little light on what part is played by the long continued losses of blood by the horses.

Putting together these results we feel justified in drawing the conclusion that bleedings of the frequency recorded may acutely terminate the life of a horse if he has been under routine treatment in antitoxin production for a sufficient length of time to give rise to extreme amyloid degeneration of a portion of the liver. Attention to this point might lead to a practical saving by prolonging the life of the valuable animals.

III. *Pathological anatomy.*

Gross examination. — Important gross pathological lesions having any bearing on the present problem are limited to the spleen, liver, and peritoneal cavity.

The peritoneal cavity in the two horses that died was found to contain large quantities of dark blood-stained serum and uncoagulated blood. Large portions of the liver were found to consist chiefly of blood clot. Margining the clots were areas of liver tissue intermingled with smaller clots and portions of liver in which the structure is roughly preserved but with a diffuse infiltration of blood. Surrounding the clots and in portions not hemorrhagic there are areas in which the liver substance is very pale and waxy in appearance. The tissue is very friable. In one horse large masses consisting of a large connective tissue capsule and a central content of dark grumous fluid, were found. The liver is covered by the adherent omentum. On cut section the vessels are wide open and patulous.

Microscopic examination. — The material for histological examination came from the horses listed in the table. The horses unaffected have been considered as control material in a general way. In addition to these I have been able to examine the tissues of a number of horses recently autopsied in Montana under the supervision of Dr. Smith. These control studies have made it possible to limit the present description to the relatively gross changes — amyloid degeneration and hemorrhagic necrosis. All the more minute lesions and alterations are found in horses outside the antitoxin series as frequently as in it.

The autopsies have been performed as quickly as possible after death. One horse died in the night and the post-mortem was held eight hours later. In most cases the animals were killed and the section made at once.

Zenker's fluid has been the routine fixative. All the tissues have been examined fresh or after formaldehyde fixation for fat, staining with Sudan III. in the later case. The liver in two instances was fixed in absolute alcohol and stained for glycogen.

The routine stains used have been the eosin-methylene blue combination according to Mallory and Wright, and hematoxylin and eosin. Amyloid was recognized by the gross appearance and under the microscope by its optical characters, distribution, general staining reactions and, in some instances, by the specific stains on fixed tissues. The reactions to iodine, iodine followed by sulphuric acid, and to methyl violet have been readily obtained and have been quite permanent after Zenker's fixation. The reactions, however, are seldom obtained throughout the sections. Mallory's connective tissue stain and Von Gieson's picro-acid fuchsin followed by hematoxylin have been found most useful in determining the extent and exact distribution of the amyloid degeneration. All permanent section work was done after embedding in paraffine.

The tissues of the horse have been studied but little by modern histological methods. The frequency of lesions due to worms, the common presence of glycogenic infiltration of the liver, and the impossibility of fixing the blood in the vessels or studying it in unfixed material before it has undergone profound alterations are features so confusing that we think it worth while to consider them in detail.

There is an apparent increase in eosinophils in the tissue generally. This is especially prominent in the spleen and some lymph nodes. There are also local areas of connective tissue overgrowth in the lung and mesentery of some of the horses of our series which show enormous numbers of these cells. These are changes due to the invasion of the tissues with Sclerostomum equinum.

The livers which are normal on gross appearance and those least damaged by hemorrhage and amyloid degeneration, when fixed in Zenker's fluid and stained by any of the methods used, give an unusual picture. The cells are swollen and have but little body. The cytoplasm is scant and coarsely granular. Definite vacuolation is not present, but in some instances a heavy cytoplasmic reticulum is seen. The cell wall is heavy and the nucleus stains lightly. The sinusoids are narrowed by the swollen cells. The changes in the parenchyma are probably those of a pronounced glycogenic infiltration. Fatty degeneration has so rarely been encountered that it could not be responsible. Glycogen has been very abundant in the cases in which it has been tested for. The changes are more pronounced in our own horses, grain-fed and kept quiet, than in the range horses of the Montana series. There are changes in the blood within the vessels which are best considered along with similar changes in the spleen, adrenal, and kidneys.

In the spleen of all the horses there are focal areas of intense congestion and hemorrhage. These areas are usually in the pulp near the trabeculæ. They are frequent in the trabeculæ, and are nearly always found to some extent in the capsule. In the cases in which the structure is nearest normal these areas are relatively few, perhaps one or two to a section and they show only congestion with dilatation of the sinusoids. To these may be superadded diffuse hemorrhage into the pulp. In one of our own horses the connective tissues in these hemorrhagic areas show hyaline necrosis and there is an infiltration of the area with polymorphonuclear leucocytes. The small acute hemorrhages without tissue reaction may perhaps be developed by a contraction of the spleen during the death agony. The trabeculæ in the horse's spleen are very heavy and contain a great deal of involuntary muscle.

When the venules associated with these areas appear in sections they are usually found to contain a dense clump of red blood corpuscles. This clump of erythrocytes may extend into neighboring sinusoids. The clumped corpuscles stain with greater than normal intensity with eosin and phosphotungstic acid hematoxylin. They often seem to be held together by some extracellular substance, but the outline of the cells is usually distinct and can always be made out. Typical hyaline thrombi have not been found in the spleen.

Similar dense clumps of corpuscles, and all transitions to them from the normal isolated corpuscles, are found occasionally in the kidney, and nearly always in the adrenal. In the latter organ they are usually unassociated with parenchymal or interstitial lesion. In the case of horse No. 93, a normal horse bled repeatedly, one adrenal shows such clumping in extreme degree in the periphery of the medullary portion. Here there are many hyaline thrombi, and a transition to the hyaline thrombus from the fused mass of red blood corpuscles is indicated. But whenever there are loose corpuscles or small clumps of corpuscles which can still be recognized as such there are also large numbers of coarse granules, possibly platelets. When the hyaline thrombus is well formed these are no longer to be seen. What becomes of them or why they disappear is uncertain. It is not impossible perhaps. that they should flow together to form an imbedding substance for the red blood corpuscles. Associated with this thrombosis of the medullary veins is an intense congestion of the inner cortical zones. The opposite adrenal shows no acute lesion, but the remains of some hyaline thrombi almost absorbed are found in a few vessels. In the liver the sinusoids are frequently filled with irregular shadows varying from one-fourth to twice the size of the red blood corpuscle. They seem to be of erythrocytic origin. The better preserved erythrocytes have a tendency to clump and are sometimes fused. These changes are probably not an artifact due directly to the fixing fluid. They are present in tissue fixed in Zenker's fluid — formalin — acetone, methyl alcohol and absolute ethyl alcohol. The large and small shadows and the closely clumped hemoglobin containing red blood corpuscles have been seen several times in fresh specimens of these livers teased in normal salt solution. The changes in the blood in the liver sinusoids are more pronounced in the tissues of our own horses than in those of the western series, and this in spite of the fact that in certain instances in our own cases especial effort was made to get quick fixation of small blocks.

It is probable that the horse almost always has in the spleen, and frequently in other organs, small red blood corpuscle plugs in the capillaries and small veins, and that under favorable conditions these may progress to the formation of hyaline thrombi and give rise to lesions of microscopic or small macroscopic size. It is possible that this tendency to intravascular clumping has relation to the rapidity with which the horse corpuscles clump and settle in horse serum either diluted or undiluted outside the body. It seems impossible at the present time to decide which of these changes are due to post-mortem, and which to antemortem conditions except when the hyaline thrombus is fully developed or when there is some definite lesion of the surrounding tissues.

These microscopic thrombi and agglutination clumps and their relation to the focal lesions revealed by the microscope are of especial importance in the consideration of the pathological condition at present under discussion because of the frequency with which Pease and Pearce have observed thrombi in the animals of their series. In our own cases gross thrombi have not been found and it would modify our conception of the etiology of the condition if the microscopic examination warranted the assumption that there was an extensive thrombosis of the small vessels. This, however, is not the case.

The gross examination leads one to the conclusion that the important constant pathological changes in the immunized and bled horses are localized in the liver and spleen, particularly the liver. Careful microscopical examination of the tissue from the animals, controlled by examination of a number of animals untreated or treated with wholly different agents, confirms this opinion.

The most striking of the essential lesions histologically, as well as macroscopically, are found in the liver. In the tissue of the five horses of our series which show abnormalities in the liver, it is possible to trace a series of changes beginning with slight amyloid degeneration which becomes extreme, a subsequent and probably consequent period of active destruction, diffuse and massive destructive hemorrhages with cellular, lobular, and multilobular necroses, and finally, reparative processes affecting both interstitial tissues and parenchyma.

Of nine animals treated with toxin for a long time and bled repeatedly, which died or were killed, five show amyloid degeneration of the liver. One horse (71) shows only scattered small masses affecting single endothelial cells. In four the amyloid is in large amount. It affects in most important degree the outer one to two-thirds of the lobule. In the extreme cases small irregular extensions may reach to the region about the central vein. In every case the amyloid is placed in close relation to the endothelial cells lining the sinusoids. The line of division between cells can be traced through the mass of amyloid. There are thus formed units of the amyloid mass corresponding in size, and at first in position, to the endothelial cells affected. These units can be distinguished even where the degeneration is extreme and they become closely massed together. The nucleus of the endothelial cell and such parts of the membrane as remain visible, are always to the side toward the lumen of the vessel and are closely adherent to the amyloid. There is usually a well marked space between the outer surface of the amyloid masses and the adjacent liver cells. Projecting into this space there is frequently a radiating fringe of amyloid (see figure).

The space taken by the amyloid is gained at first by the atrophy of the liver cell. Later the sinusoid is encroached upon, but even when the amount of amyloid is extreme and the liver cell cords are reduced to a thin line the sinusoid is often patulous. Finally however, — and this is frequent in the cases showing hemorrhage, — the blood channels are wholly obstructed. The very narrow and obstructed sinusoids are only found at the periphery of the lobule.

The hemorrhagic areas of the liver usually show a large mass or masses of laminated clot intermingled with and bordered by fragments and lobules of liver tissue in various stages of necrosis and disorganization. Except in the late cases when repair has begun there is never any well-defined cellular reaction in these border zones. Some of the border lobules are broken entirely away, others remain connected with the more normal liver tissue. Some show complete hyaline necroses. Others are diffusely infiltrated with blood and are partly necrotic. In one case well defined lines of hyaline central necrosis extend from the hemorrhagic to the more normal areas.

Usually there is no indication whatever of the path of the hemorrhage. But in the case of horse 3, in the regions somewhat removed from the gross hemorrhage, but where the amyloid degeneration is very marked, there frequently are found lobules whose sinusoids are occluded in the outer third. Many of these show diffuse hemorrhages into the central portions. Others have a much dilated, very thin walled central vein. The expansion of the vein may be sufficient to compress the central portion of the cords of cells back against those that are supported by the mass of amyloid. Occasionally the central vein is ruptured.

In one horse (No. 30) areas of old hemorrhage are found with the recent ones. In these cases the clot is wholly or partly organized, giving rise to areas of moderately dense cellular and fibrous connective tissue. In the spaces of the latter are found many endothelial cells containing blood pigment, and moderate numbers of wandering cells of other kinds — lymphocytes, plasma cells, and eosinophiles.

So far as my observation goes, the connective tissue growth always takes place from the periportal region. It is frequently possible to see areas of laminated clot touched on one side by apparently healthy liver tissue and, growing into it from another point, young connective tissue and blood vessels. On the side of the liver tissue there is no reaction whatever. The endothelial cells of the sinusoids or cells from the center of the lobule do not seem to take part in the process of repair.

Bile-duct regeneration or proliferation is found in this new connective tissue growth. In these cases, too, there is an evident attempt to regenerate liver parenchyma. Irregular masses of cells having the character of liver cells, but without definite relation to sinusoids are found near the proliferating connective tissue. Occasional cells among these show definite karyokinetic figures. In a few places in the case of horse 3, where the amyloid degeneration is extreme but where there has been no hemorrhage, there is a proliferation of capillaries within the mass and these are followed by an increase of connective tissue. The process is not extensive enough to show the end result.

In one case (No. 3) there are in addition to amyloid degeneration very small focal hyaline hemorrhagic necroses. They seem to be gradually replaced by round cells and it is not likely that they influence the general disease, but they are interesting as being the only lesions found in these livers resembling in any way the lesions of acute diphtheria.

The spleen in two cases shows amyloid degeneration. In one of them the areas affected are small. They lie in the pulp just outside the Malpighian bodies. The degeneration, here as in the liver, seems to affect endothelial cells lining the sinusoids and these show little tendency to fusion. In the second case the degeneration is extreme, the whole pulp being transformed. The Malpighian bodies remain as rather narrow lines and small collections of cells. The sinusoids, not to be followed in section, must have been open, as they contain well preserved red blood cells.

The other organs show neither important nor constant lesions. A few of the glomeruli in the kidney of one horse show amyloid degeneration.

SUMMARY AND CONCLUSION.

Horses may be kept for a long time producing antitoxin if they are not bled more severely than five liters per one thousand pounds each three and one-half to four weeks. After the treatment has continued long enough to develop amyloid degeneration of the liver, increasing the frequency of the bleeding risks killing the animal. Some saving may result from the practical application of this knowledge to routine work.

Amyloid degeneration of the liver and less frequently of the spleen is produced in a majority of horses by the routine treatment with diphtheria toxin and repeated bleeding extending over a period of three years. The longer the treatment is continued the greater the certainty of the onset of the amyloid disease.

Horses with amyloid degeneration of the liver are very subject to hemorrhage into the liver substance and to rupture of the organ, with hemorrhage into the peritoneal cavity.

The hemorrhage extends more readily because of the great friability of the amyloid liver. It very possibly results from an extension of intralobular hemorrhagic infarctions which are probably directly induced in the already diseased liver by the disturbances in circulation incident to bleeding. When the hemorrhage does not break through the surface it is either encapsulated, resulting in a blood cyst, or absorbed and organized, resulting in an irregular increase in connective tissue in the liver. Regeneration of parenchymal and bile duct epithelium is attempted in the late cases.

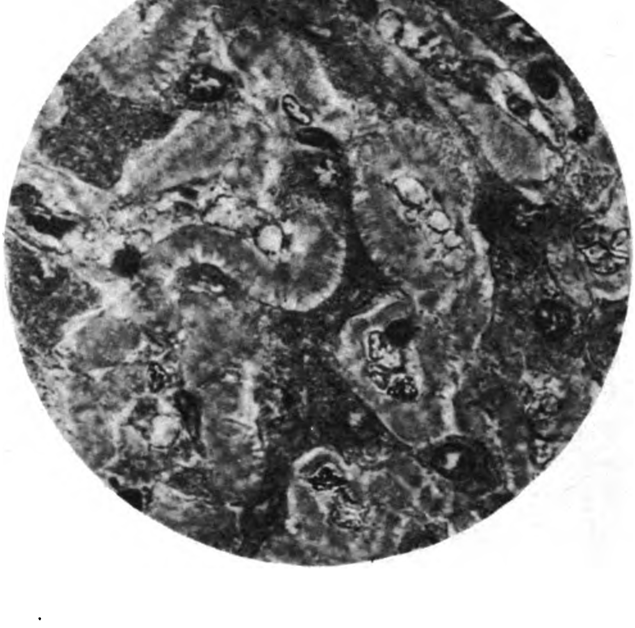
NOTE. — When this manuscript was prepared I overlooked the fact that the paper of Pease and Pearce referred to in the text (p. 451) had been published in The Journal of Infectious Disease, Vol. III., No. 4, June, 1906. They report the death of ten horses with rupture of the liver and hemo-peritoneum, and three horses killed at some stage of immunization. The horses had been treated with the toxic products of four species of bacterium and the results are with justice ascribed to the bacterial products, rather than the antiseptics and bouillon constituents which indeed could be definitely excluded as factors in several of their cases. The lesion underlying the liver rupture in all their cases was an acute general or local necrosis of the organ. This radical difference between their results and ours seems to depend on a difference in methods employed. Their treatment of the animals is evidently more vigorous than that employed in our laboratory. The doses of toxin are larger and the bleedings more severe. As a consequence, the animals do not live so long and the essentially chronic type of fundamental lesion seen in our cases does not have time to develop. They note two cases of amyloid degeneration, both in horses treated with diphtheria toxin. They are inclined to attribute this degeneration to the influence of the many abscesses which these horses suffered. While the abscesses may have hastened the process, our experience rules them out as an essential condition. Abscesses have been very rare with us. Venous thrombi — sometimes in the portal vein — were found in a number of their cases. They hesitate to draw the conclusion that the necroses were secondary to the vascular lesion. In this connection the frequency of thrombi in the blood vessels of the horse due to sclerostomum equinum should be noted. They note that in one of their cases death occurred immediately after bleeding. No information is given on this point in connection with the other cases. The case strengthens our idea that the disturbance of circulation incident to bleeding may induce the liver rupture when the anatomical foundation is laid. On the whole, the results in the two series of cases are complementary.

DESCRIPTION OF PLATE XXIX.

AMYLOID DEGENERATION IN LIVER OF HORSE 3 (x 1,000 diameters).

The amyloid lies close to the sinusoids. There is a narrow space between the amyloid and liver cells with fine radiating prolongations of amyloid reaching into it. The sinusoids contain shadows of variable size and density.

[We thank Dr. S. T. Orton of the Boston City Hospital, who took the photograph for us.]



Lewis. Antitoxin Hepatitis

DIPHTHERIA–TOXON PARALYSIS IN THE GUINEA–PIG.*

PAUL A. LEWIS, M.D.

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Since the organization of the antitoxin laboratory eleven years ago the record of every guinea-pig used in the routine tests of the strength of diphtheria toxins and antitoxins has been kept, from the time of its birth or purchase until its death or sale. The records include as completely as circumstances will allow the genealogy of the animal, its particular treatment and the result of the treatment. It has been in the mind of Dr. Theobald Smith, the director, for some time, to study over these records for the facts which might be contained in them regarding the paralysis which affects a certain number of the animals surviving the treatment. A part of the records had been tabulated with this in view when I was offered by him the opportunity to undertake the outlined study. The following paragraphs are presented with the hope that the organization of this material may be partly worthy of the system on which it has been collected.

As is now well known to every one who has treated guinea-pigs with mixtures of diphtheria toxin and antitoxin according to the method devised by P. Ehrlich, a certain proportion of the animals which survive the inoculation for three weeks or longer suffer from paralysis during the third or fourth week. The paralysis may be slight, affecting but one limb, or somewhat embarrassing the respiration, or it may be severe and cause sudden or gradual death. It may be transient, lasting from a few days to two weeks or it may, as has been said, terminate fatally at any time. The paralysis is never permanent. Ehrlich in his fundamental work on the constitution of diphtheria toxin reached the conclusion that the crude toxin was not as had been previously assumed a single substance, but was essentially a mixture of a number of distinct poisons more or less closely related in their origin,

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disease producing power, and affinity for diphtheria antitoxin. The paralytic phenomena he supposed to result from the specific action of a portion of the crude toxin called by him toxon. The repetition of some fragments of Ehrlich's classical paper may not be out of place here, giving as they do a concise statement of his views.

> "In my second research, 'Ueber die Constitution des Diphtheriegiftes,' I supposed that we had to do with a primary secretive product of the diphtheria bacillus which I called 'toxon.' The toxon possesses the same haptophore groups as the 'toxin,' but a lower affinity for the antitoxin. The main difference lies in the toxophore groups, in that the toxon even in very large doses does not cause death, but paralysis which only comes on after a long incubation of fourteen days or more."[1]
>
> "In contrast to this (the action of toxin) the toxon, that is, the serum-poison mixture in which only the toxin fraction is fully saturated, never kills acutely even in very high doses. The property of causing inflammation may be entirely wanting in small doses and in large doses is present in much diminished grade. The edema disappears completely in the course of a few days, necrosis is absent, and loss of hair, while common, is only to be seen in the form of a partial baldness. On the other hand, the paralytic phenomena which develop between the fourteenth and twenty-eighth day, according to the dose, usually in the third week, are characteristic. In these animals there is often no trace of local reaction, they retain their body weight and are then suddenly stricken with paralysis, to which they may succumb in a few days. I have never observed such a result with animals injected with diphtheria toxin alone."[2]
>
> "The quantity of toxon in proportion to the 'toxin' may vary according to previous experiments from 0 to 300 per cent."[3]

Our animals available for this study must be divided according to the treatment received into three classes.

(*a.*) Guinea-pigs injected with toxin only. — Ehrlich, as above quoted, states that he had never seen paralysis in an animal injected with toxin only. Dreyer and Madsen had, however, worked with a toxin which when injected in appropriate doses caused such paralysis. Analysis by the Ehrlich method of toxin-antitoxin mixtures showed that there was present in this poison fifteen times the usual amount of toxon.

We have recorded (to May 15th, 1906) two hundred and twenty-one guinea-pigs injected with toxin only, which survived four weeks. In seven of these, or three and one-tenth per cent, paralysis was noted. As four of these cases occurred a number of years ago when the subject had received little attention, the accuracy of these observations has recently been considered doubtful. During the past winter we have made and tested the strength of a considerable number of toxins using slight modifications in the method of preparing the bouillon. Among the pigs surviving the injection of these poisons three cases of paralysis have occurred. One of these while persistent was slight. In another the necrosis while not especially severe persisted to the time when paralysis developed and the animal emaciated so that there was some chance for confusion. The third case, however, was as typical as could be wished for. The protocols of two of these cases are presented.

I. *Guinea-pig No.* 4245.

March 28, 1906. Weight, 254 grams.

3 P.M. Diphtheria Toxin Lot 16 R.

Diluted ½ cubic centimeter toxin + 199½ cubic centimeters salt solution.

Injected 2.4 cubic centimeters toxin diluted = .006 cubic centimeter toxin.

March 31, 9.30 A.M. Moderate edema; quiet.

April 3, 9.30 A.M. Moderate, poorly defined, soft induration; active.

April 7. Slough one inch diameter; active.

April 10. Slough one inch diameter; active.

April 12. Slough three-fourths inch diameter; emaciation noticeable.

April 16. Ulcer one-half inch diameter with thick scab. Animal emaciated. Animal has irregular respiration. Weak in fore limbs.

April 20. During past days has developed a well-marked paralysis of fore limbs. Abdominal muscles are weak. Hind quarters relatively strong.

April 21, 9 A.M. Same condition. Seems in fairly good shape.

10 A.M. Found dead. Autopsy. Ulcer .5 centimeter diameter in left abdominal region. No definite gross lesions of any organ.

II. *Guinea-pig No.* 4292.

May 9, 1906. Weight, 253 grams.

12 noon. Diphtheria Toxin Lot 16 Q.

Dilution ½ cubic centimeter toxin + 199½ cubic centimeters salt solution.

Injected 2.4 cubic centimeters toxin diluted = .006 toxin.

May 12, 9.30 A.M. Thin edema, one by two inches; active.
May 15, 12 noon. Thin irregular superficial induration; active.
May 20. Superficial ulcer three-fourths inch diameter. Large bare spot; active.
May 28. Superficial ulcer one-fourth inch diameter. Well marked paralysis of hind limbs, particularly the left.
June 4. No local lesion. Paralysis still same.
June 12. Paralysis still severe.
June 19. Paralysis much improved.
June 28. Paralysis has disappeared. Animal fairly strong and active.
June 29. Weight, 345 grams.

The minimum fatal dose of these toxins was not more accurately determined.

All of the toxins which produced paralysis and most of those tested have been made with the well-known culture isolated by Park and Williams. The bouillon has in all cases in which paralysis was produced and in most others been prepared from beef by the fermentation method.[4] Under these conditions the minimum fatal dose of the toxin obtained has varied from .002 cubic centimeter to .01 cubic centimeter ++. The quantitative variation in toxon cannot be stated, but the certain although infrequent development of paralysis in animals injected with toxin only shows, either that the usual amount is sometimes far exceeded as an absolute quantity or that there is a fixed quantity present which is occasionally demonstrated, because the toxin fraction is unusually weak. If the latter supposition were true, nearly all of the animals surviving the dose given the guinea-pigs, which became paralyzed, should have shown toxon effects. The dose (.006 cubic centimeter) has been the usual one for the surviving animals, and was that given the animals in question. We therefore conclude that there is a great variation in the absolute quantity of toxon developed by the same culture of B. diphtheriæ under conditions encountered in toxin production. It is interesting that the only toxins showing effective amounts of toxon were very weak in the acutely acting substance, but we have no data extending this observation.

The larger number of our animals have been injected with mixtures of toxin and antitoxin in testing the potency of the serum. For this purpose a strong toxin is chosen and kept for some time in the dark at a temperature of fifty to fifty-five degrees Fahrenheit. At present these poisons are kept for at least a year before being used. Decline in toxicity and in affinity for antitoxin is rapid when the toxin is fresh, but by the end of a year the composition is relatively stable so far as the toxin fraction is concerned. (The toxin is now carefully standardized as to L_+ and minimum fatal dose. Comparatively few such toxins have been used.) There are no data on the condition of the toxon beyond the fact that Donitz[5] working under Ehrlich showed that their poison V. lost eight-tenths of its toxon equivalents in three weeks, during which time the L_+ dose remained the same, and if this represents a constant change in crude toxon the "toxon" should become at least as stable as the "toxin" at the end of the year.

(*b.*) Guinea-pigs injected with toxin antitoxin mixtures containing one-tenth the standard antitoxic unit which neutralizes theoretically ten minimum fatal doses.

Using these relatively stable standardized toxins in mixture with antitoxin, we had two hundred and nine surviving animals during the period when the injected dose contained about one-tenth the standard unit of antitoxin, and toxin approaching the point of neutralization. Of these, nine, or four and three-tenths per cent, became paralyzed. At first glance it would seem that the injection of the increased quantity of toxin with the antitoxin had been without effect on the incidence of paralysis. But the toxins used in this series of tests if used alone would probably almost never produce paralysis, and the demonstration of toxon in them is due to the injection of the larger quantity of poison in which the toxin fraction is in large part neutralized by the antitoxin.

(*c.*) Guinea-pigs injected with toxin antitoxin mixtures containing the full standard antitoxic unit which neutralizes theoretically one hundred minimum fatal doses of toxin. (The unit neutralizes approximately forty minimum fatal doses of our test toxins.) According to the present system of testing, the unit of antitoxin, neutralizing theoretically 100 m. f. d. of toxin, is injected and in tests of serum of unknown strength an effort is made to inject as nearly as possible this quantity of antitoxin. It was in the development of this method that paralysis began to be noticed on a large scale, and it is since its general introduction that the late lesions of the nervous system have been commonly noticed in the guinea-pig. While using this system we have had eight hundred and fifty-eight surviving animals, of which two hundred and fifty-nine (or thirty and one-tenth per cent) have suffered paralysis. I have endeavored to analyze the data on the animals treated in this way, so as to show the influence of the particular toxin used, and the varying susceptibility of the guinea-pig, on the incidence of paralysis.

I. INFLUENCE OF TOXINS.

The facts in regard to the test toxins, the culture used in their production, the period of growth, the age of the toxin when first and last used, the method of preservation before and during use, the number of surviving animals and the percentage paralysis are presented in the accompanying table:

TABLE I.

Relation of paralysis to toxin used.

Test toxin number.	Culture used.	Date of preparation. Time incubated.	Age in Months.		Method of preservation.	Number of animals injected.	Number paralyzed.	Percentage paralysis.	Total percentage paralysis for toxin.
			When first used.	When last used.					
5	Park and Williams.	Nov., 1897. 10 days.	1 mo. +	2 mo. +	Single dose bottles.	5	3	60% (winter)	Toxin 5 = 60%.
6	14	Feb., 1898. 16 days.	2	4	Single dose bottles.	24	2	8⅓% (summer)	Toxin 6 = 8⅓%.
7a . . .	14	June, 1898. 9 days.	1		Single dose bottles.	19	3	15.7%	
7b . . .						2	1	50%	
7c . . .				6		20	7	35%	Toxin 7 = 26.8%.
8	14	Oct., 1898. 10 days.	8	24	Single dose bottles.	22	9	40.9%	Toxin 8 = 40.9%.
9	Park and Williams.	Oct., 1898. 8 days.	2	24	Single dose bottles.	87	34	39.1%	Toxin 9 = 39%.
10a . . .	Park and Williams.	Nov., 1899. 7 days.	7	12	Single dose bottles.	47	20	42.5% (summer)	
10b . . .			14	16	10b bottles had toluol added.	25	13	52% (winter)	
10c . . .			16	24		43	15	34.8% (summer)	
10d . . .			24	25		14	6	42.8% (winter)	Toxin 10 = 41.5%.

TABLE I.— *Continued.*

Test toxin number.	Culture used.	Date of preparation. Time incubated.	Age in months.		Method of preservation.	Number of animals injected.	Number paralyzed.	Percentage paralysis.	Total percentage paralysis for toxin.
			When first used.	When last used.					
11a . . .	Park and Williams.	Feb., 1901. 7 days.	11	17	Single dose bottles.	50	26	52%	
11b . . .			17	27		57	18	31.5%	Toxin 11 = 41.1%.
12a . . .	Park and Williams.	Feb., 1903. 7 days.	4	9	Single dose bottles.	40	15	37%	
12b . . .			8	9		12	1	8⅓%	
12c . . .			8	20	12c bottles had toluol added.	135	16	11.8%	Toxin 12 = 17.6%.
13b . . .	Park and Williams.	Nov., 1903.	12	18	Used repeatedly from large bottle.	35	9	25.7%	
13c . . .			18	24	Toluol added.	53	9	16.3%	Toxin 13 = 20%.
14a . . .	Park and Williams.	Sept., 1904.	12	18	Used repeatedly from large bottle.	102	24	23.5%	
14b . . .			18	21	Toluol added.	44	13	27.2%	Toxin 14 = 25.3%.

NOTE. — Sub-letters under toxin indicate order in which separately stored parts were used.

The table shows a variation of from eight and one-third to sixty per cent in the amount of paralysis produced by the different toxins. It will be noticed that the extremes occurred when fresh toxins were used and if allowance is made for the influence of season, they probably represent differences in "toxon" content of the crude toxin. In support of this view is the fact that toxin No. 5, giving the highest percentage paralysis, also produced paralysis once when injected alone. The variation in the number of cases of paralysis with the toxins numbered 8 and above is influenced by other factors and probably throws but little light on the composition of the toxin. Taking account of the influence of season by balancing the summer and winter injections with toxin 10 — and disregarding toxin 14, the records of which are incomplete, there is indication of a moderate progressive decrease in the production of paralysis as the toxin gets older. It is also to be noted that the toxins 12, 13, and 14 (to date) gave about half the total percentage paralysis of the toxins immediately preceding them. That this represents altogether a difference in composition may be doubted. Beginning with toxin 12, toluol was added to the vials or bottles in use, to prevent evaporation. Since 1903 also many guinea-pigs of increased resistance to toxin have been used and this has probably tended to decrease the paralysis under these toxins. Exact value cannot be assigned to these factors.

II. THE SUSCEPTIBILITY OF THE GUINEA–PIG TO PARALYSIS.

(*a.*) Influence of season. — The susceptibility of the guinea-pig to paralysis is somewhat greater in winter than in summer. This was shown definitely by charting the eight hundred and fifty-eight animals surviving treatment with toxin-antitoxin mixtures containing the full unit of antitoxin, according to the month in which the injection was made. Table II. shows the results of the calculation for each month and grouped into seasons, as summer and winter. The difference in resistance is probably dependent on feeding. Our animals receive green grass during the summer months and this is replaced by carrots in the winter. The better ventilation in the quarters during the summer may also be a factor.

TABLE II.

Influence of Season.

Injection made in	Number injected.	Number paralyzed.	Percentage paralysis.	
November .	58	23	39.6%	
December .	66	21	31.8%	
January . . .	89	33	37.0%	
February . .	75	25	33.3%	
March	71	21	29.9%	
April	81	19	20.9%	Average (6 months), winter, 32%.
May	74	22	29.7%	
June.	67	18	26.8%	
July	59	13	22.0%	
August. . . .	69	23	33.3%	
September .	72	22	30.0%	
October . . .	68	16	23.0%	Average (6 months), summer, 27.5%.

It may be possible to show a corresponding increased susceptibility to the acutely acting fraction of the crude toxin in the winter months.

Returning for a moment to the experiments with toxin alone and with mixtures on the ten m. f. d. basis it is found that thirteen of these animals of the sixteen which became paralyzed were injected in the winter months. This would indicate that when other factors are so delicately balanced that the incidence of paralysis is in doubt this seasonal influence, slight in general, might become decisive.

(*b.*) Influence of family.— It has already been shown by Theobald Smith[6] that the guinea-pigs used in this laboratory since 1903 have varied greatly in their resistance to the acutely acting portions of diphtheria toxin. This increased resistance was shown to be constant with the offspring of the same female. The variation has been so great that it has been necessary to take constant account of it in carrying out the routine work of the laboratory, and for some time the resistance ratio of the offspring of each breeding female has been carefully determined. Struck by the close maternal relationship of several of the pigs paralyzed after injection with toxin only, and faced by the difficulties of explaining a drop of one-half in the percentage of paralysis during the past three years, and with the last three test toxins used, I have classified all of the pigs which have survived treatment with the current toxin-antitoxin mixture according to their descent through the female line. Careful consideration of the tables so formulated has failed to show any evidence of a hereditary influence extending to the second generation, although the experiments are not yet extensive enough to be by any means conclusive. But if from these tables one selects the mothers, the resistance of whose offspring to "toxin" is wholly or partly determined, they can be classified in two groups. Since special attention has been given to descent in its relation to resistance to toxin we have had twenty mothers known to breed pigs of normal resistance and thirty-three breeding those of more or less increased resistance. Table III. shows the result obtained by counting, according to this grouping, the number of the offspring surviving treatment for the required length of time and those among them which became paralyzed.

TABLE III.

Influence of Family.

	Number of Females Bred from.	Number of Young Injected and Surviving 4 Weeks.	Number Paralyzed.	Percentage Paralysis.
Offspring of normal resistance to diphtheria "toxin"	20	53	27	50.9%
Offspring of increased resistance to diphtheria "toxin"	33	128	16	12.5%

NOTE.—Only guinea-pigs injected with mixtures certainly between the limits L_0 and L_+ have been used in making up this table.

The results show that fifty per cent of the guinea-pigs of normal resistance which survived doses certainly between the limits L_0 and L_+ became paralyzed. Of correspondingly treated animals whose resistance to "toxin" is known to be above normal, but twelve and five-tenths per cent suffered paralysis. The number of animals in this series bred from any one mother is much too small to warrant a conclusion that would apply to individuals, but in general it is true that an increase in resistance to "toxin" is accompanied by an increase in resistance to "toxon." Experiments directed toward that end would be required to determine whether or not the increase is present in all cases and proportional.

If the classification of the animals into groups of normal and increased resistance be maintained and each group be sub-divided to show the local lesion suffered by the paralyzed and non-paralyzed pigs of the group, the following table can be made:

TABLE IV.

Showing relation of paralysis to local lesion in guinea-pigs of varying resistance.

	Guinea-pigs of Normal Resistance to Diphtheria "Toxin."		Guinea-pigs of Increased Resistance to Diphtheria "Toxin."	
Local Lesion Suffered.	Transient Edema.	Ulceration.	Transient Edema.	Ulceration.
Number of animals paralyzed...	5	22	3	13
Number of animals not paralyzed	21	5	33	79

These animals are all of known resistance to "toxin" and all were injected with toxin-antitoxin mixtures in which the toxin fraction is incompletely neutralized as evidenced by at least a trace of local lesion. There have been five animals paralyzed which showed no trace of local lesion on the third day.

The figures show that in our series the pure toxon disease has been relatively rare. The moderate number of cases in which animals with no local lesion at all have suffered paralysis serve to show that it is possible with the conditions under which we have worked to isolate completely the toxon action. But the very considerable number (twenty-one) of pigs of normal resistance having transient local lesions but no paralysis show with equal conclusiveness that in the majority of cases the toxin quantity L_0 must be clearly exceeded before the toxon can be demonstrated with certainty. Considering animals of normal toxin resistance and supposing the period of life after injection to be four weeks, the rule could be laid down that the nearer the L_+ dose was approached the greater would be the chances of paralysis. It is impossible at the present writing to decide whether or not the animals of heightened resistance will follow this law. That about eighty per cent of the normal pigs showing ulceration became paralyzed as against the fourteen per cent of those of increased resistance showing the same lesion may indicate a difference. On the other hand, supposing toxon resistance to be proportional to toxin resistance, the probability is that the dosage has been so frequently underestimated in the case of the animals of high resistance that accurate conclusions are at present impossible.

CONCLUSIONS.

1. By grouping and analyzing the records of the guinea-pigs used in a large number of routine tests of the strength of recently prepared toxins, it is shown that the crude toxins, produced by the same culture of diphtheria bacillus under conditions which are identical within limits which it is possible to determine at the present time, differ greatly in the amount of toxon or paralysis-producing poison which they contain.

2. Using old crude toxins which have become relatively stable in composition, in mixtures with antitoxin on the present Ehrlich system, it is shown that

a. Guinea-pigs are somewhat more susceptible to the action of toxon during the winter months than during the summer. A variation probably dependent on the kind of food supplied, and possibly also on the ventilation of their quarters.

b. Guinea-pigs of increased resistance to the "toxin" fraction of the poison produced by the diphtheria bacillus also show a well marked increase in resistance toward toxon. That is, they are less frequently afflicted with paralysis than are animals of normal resistance when treated with unsaturated but non-fatal toxin-antitoxin mixtures.

3. For guinea-pigs of normal resistance at least the nearer the L_{+} dose is approached, supposing the animal to survive the necessary length of time, the greater the chance of paralysis developing.

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A CONTRIBUTION TO THE KNOWLEDGE OF THE LESIONS CAUSED BY TRICHINA SPIRALIS IN MAN.*

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Introduction. — The object of this work has been to investigate the lesions caused by Trichina spiralis in man, and to confirm in man the method of distribution already established in animals.

Historical. — The old view of Leuckart in regard to the distribution of the embryos by active boring along the connective tissue has been well refuted by Askanazy and Graham. From experiments upon rabbits, Askanazy claims that the trichina embryos are deposited in the lymph spaces of the villi of the intestines by the adult females. From there they pass through the mesenteric nodes (Virchow, Fiedler, Graham, Askanazy), and the thoracic duct into the superior vena cava and the heart.

In clots from the latter places, embryos have been seen by Zenker, Fiedler, Virchow, Graham, and Staubli. From the heart the embryos are distributed throughout the body by means of the blood stream. They have been seen in the capillaries in animals by Graham.

In man the mode of entrance of the embryos in the body is not described. As the embryos have been seen in the human mesenteric glands (Virchow and Fiedler) they probably enter by the lymph, but it is not stated in what part of the gland they appear, so definite proof is wanting. Although the embryos probably are distributed by the blood, they are not described as being found free in the blood stream.

In man the following lesions are already described.

Gastro-intestinal tract. — An acute intestinal catarrh with glandular swelling (Lorenz, Cohnheim), sometimes hemorrhagic points (Ehrhardt), and even ulcers in the mucosa with

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a surrounding inflammatory reaction (Ebstein, Lewin, Lorenz), are described. The mesenteric glands are swollen, hyperemic, and sometimes show hyperplasia (Cohnheim). The liver shows a fatty change in the parenchymatous cells (Cohnheim and others). The heart shows a fatty change (Cohnheim). In rats Graham describes areas of cell infiltration, and in man Zenker finds the embryos in the heart. The kidneys in man show a fatty change in their parenchymatous cells (Cohnheim). The spleen shows nothing abnormal except an increase of size in some cases (Cohnheim). Beneath the parietal pleura minute hemorrhagic areas exist (Tungel). In the lungs, catarrhal and embolic conditions exist (Rupprecht), also marked congestion and edema with beginning bronchopneumonia (Cohnheim). (In animals Cohnheim found embryo trichinæ in hemorrhagic areas.) Many observers mention edema of the lower extremities. The other organs and glands are not mentioned as being involved in the disease. In man the muscles first present macroscopic changes about the fifth week (Cohnheim).

The mode of entrance into the muscle fiber, and the earliest muscle lesions in man are not described, but in animals are extensively described by Leuckart, Graham, Soudakewitsch, and others. In man the muscle changes are described during the fourth week by Lewin and Soudakewitsch, during the third week by Tungel and Ehrhardt, and during the second week by Lorenz.

The embryos first lie stretched out in the muscle fiber and become spirally twisted as they grow (Lorenz, Soudakewitsch). (They reach their full development about ten days after entrance in animals (Leuckart).)

With the growth of the embryo the muscle fiber degenerates. The nuclei increase in number by direct division, and lie with a small amount of protoplasm in the finely granular mass of degenerated muscle protoplasm (Lewin). This surrounds the enrolled trichina.

Surrounding the invaded fiber and scattered diffusely throughout the muscle is an inflammatory reaction (Lorenz and others). The muscle fibers in general not invaded by the trichinæ show changes. The muscle nuclei are increased (Fiedler), and a vacuole degeneration is present (Nonne and Hoepfner, Soudakewitsch). The muscle also seems to have a predisposition towards hyaline degeneration (Volkmann, Soudakewitsch).

In the formation of the capsule the sarcolemma plays an important part (Virchow, Lorenz), and the degenerate muscle mass also (Lorenz). The capsule is closed at the ends by the activity of the muscle nuclei. Connective tissue does not take part in the capsule formation (Lorenz).

Volkmann describes fibrillæ surviving within the degenerate protoplasm and in the capsule. Lorenz does not find them.

After encapsulation the interstitial process quiets down with the formation of scar tissue. Volkmann claims he has never seen regeneration of muscle. Zenker claims he has, and Lorenz sees a suggestion of it. Finger reports regeneration of size and return of function.

Some of the encapsulated trichinæ may be destroyed early by invasion and absorption (Ehrhardt). The ultimate fate of the majority is described by Langerhans, Lorenz and others.

It remains, therefore, to demonstrate the position of the Trichina embryo in the lymph glands; to demonstrate them in the blood stream in man; to find any undescribed lesions; and to confirm those already described.

The following case, which came to autopsy at the Boston City Hospital, throws some light on these points.

The patient died twenty-six days after she claimed her illness began. The autopsy was thirty-three hours postmortem.

The gross lesions are as follows:

Gastro-intestinal tract shows minute whitish specks on the mucous membrane of the duodenum and upper jejunum especially upon the rugæ.

The mesenteric lymph nodes are enlarged, softened, and reddened.

The heart and kidneys are negative and show no fat with Scharlach R.

The liver with Scharlach R. shows large drops of fat at outer half of the lobules, and small drops elsewhere. In the feces are adult female trichinæ in great numbers, one or two in each drop, with partially developed embryos within them.

The following lesions appear upon microscopic examination. The tissues were preserved in Zenker's fluid, mounted in paraffin and stained with eosin and methylene blue. The intestines and muscles were also mounted in celloidin and different stains used. The intestines show a marked postmortem desquamation of the mucous membrane. All the coats of the intestines are somewhat edematous. Mono- and poly-nuclear eosinophiles are present in all layers. No trichinæ or embryos are found in the sections examined.

The mesenteric nodes show a fair number of eosinophiles usually in the lymph spaces. Also there are some phagocytic cells and an occasional endothelial cell in mitosis. Free in the lymph sinuses, in a section 0.6 x 0.4 centimeter, are parts of two different trichina embryos (Fig. 1 shows one).

The lungs show marked congestion and edema with considerable pigmentation near the blood vessels. In many areas hemorrhage has occurred into the lung tissue. In one section 1.0 x 1.5 centimeters, parts of four different embryos are seen in such areas free in the hemorrhage (Fig. 2 shows such an area).

The heart shows a slight general edema. In a section 1.0 x 1.3 centimeters, there are nine areas where there is cell infiltration, and necrosis of muscle with invasion of the necrotic muscle by cells. The invading cells are endothelial cells, neutrophilic and eosinophilic polymorphonuclear leucocytes, mononuclear eosinophiles, and cells of the lymphocytic series. Several of these areas in this section show part of a trichina embryo lying outside the vessels and muscle fibers (Fig. 3).

In sections of the liver 1.0 x 1.0 centimeter, sometimes no or one area of cellular infiltration exists, with necrosis of the parenchyma. The infiltrating cells are similar to those described in the heart. In one of these areas an embryo is seen outside of a vessel. In one section an embryo also lies free within a sinusoid with no surrounding reaction (Fig. 4).

In the pancreas, in a section 1.0 x 1.5 centimeters, there is one area of cell infiltration with necrosis of the parenchyma. In this area is an embryo trichina outside a vessel. The infiltrating cells are similar to those in the heart. The interstitial tissue is somewhat edematous. Rather frequently an eosinophile is seen in the connective tissue.

The kidney shows marked intracapsular and intratubular and slight interstitial edema. The vessels of the pyramids are congested.

In the bladder wall, besides some edema of the muscular coats, eosinophiles are frequently seen and a slight increase in lymphocytes occurs around the vessels. The mucous membrane shows marked post-mortem desquamation. The spleen shows besides moderate congestion, an increase in lymphocytes and eosinophiles in the blood sinuses. The ovary, adrenal, uterus, posterior nerve root ganglion and thyroid all appear normal except for slight edema or congestion or the presence of an occasional eosinophile.

In the cerebrum many areas of slight hemorrhage into the brain substance occur, around which there is no marked cellular reaction. In sections averaging 1.0 x 1.0 centimeter, there appear sometimes one, two, or no areas of cellular infiltration. The cells are chiefly endothelial and neuroglia cells with an occasional leucocyte or lymphocyte. In such an area, part of a trichina embryo sometimes appears outside the vessels. Around these areas the neuroglia shows a slight reaction as is brought out by the phosphotungstic acid hematoxylin stain (Fig. 5).

In the muscles in this case appear the following changes :

Numerous trichinæ spirally rolled, one, occasionally two in a capsule, are present in muscle fibers. In a cross section of muscle, measuring 0.3 x 1.2 centimeters, forty-five encapsulated trichinæ were counted.

The capsule surrounds the whole of the invaded fiber and is formed of connective tissue. (Sections from older cases show that this capsule contracts down to a long or short oval form.) This is proved by the fact that its fibrillary character can be demonstrated in places and that it stains like connective tissue with the various differential stains. The invaded muscle fiber undergoes a form of degeneration. The nuclei are increased in number and no longer remain at the periphery, but are scattered irregularly throughout the granular mass. The cytoplasm loses its striations and becomes a finely granular mass which is denser around the nuclei. The fibrillæ begin to disappear and gradually all go except a single layer of them at the periphery, which lie outside of the muscle nuclei and just inside of the connective tissue capsule. Spirally rolled in the center of this degenerated muscle mass lies the parasite.

Besides the degenerated muscle fibers containing grown trichinæ other muscle fibers are affected. In some the nuclei are increased, in others necrosis with invasion by phagocytic cells has taken place, in many others irregularly shaped vacuoles are formed, extending some distance along the muscle fiber which otherwise appears normal (edema).

In the muscles affected a moderately marked general edema and a diffuse interstitial process are present. The interstitial process consists of a cellular infiltration with cells of the lymphocytic series, endothelial cells, polynuclear neutrophiles and eosinophiles, and mononuclear eosinophiles. New connective tissue is also formed in places. This inflammatory process is more marked around the trichinæ and around the necrotic muscle fibers.

One embryo was found free in the connective tissue of the muscle surrounded by an area of inflammatory exudate. This apparently is a condition analogous to that described in the brain, heart, and other situations.

Some of the encapsulated trichinæ undergo degeneration. In some the granular mass is edematous, in others the cells of infiltration invade and eventually destroy the trichina and capsule and granular mass.

Discussion. — The claim (Lorenz and others) that the capsule is formed from sarcolemma and the degenerate muscle mass, that it surrounds only part of the invaded fiber and is closed at the ends by the activity of the muscle nuclei must be incorrect since the capsule has been shown to be of connective tissue and in the early stages of the process to surround the whole muscle fiber. Moreover, the constant layer of striations which separates the degenerated muscle and its nuclei from the capsule argues against the claim that the degenerated muscle mass enters into the capsule formation.

From this case the following conclusions may be drawn, and new facts added to our present knowledge:

(1.) The finding of trichina embryos in the sinuses of the mesenteric lymph nodes and in liver sinusoids shows that they enter by the lymph stream and are distributed by the circulating blood.

(2.) The presence of the embryo trichinæ in the hemorrhagic areas in the lungs of man confirms the observation of this fact made in animals.

(3.) The embryos may break out of the vessels in the liver, pancreas, brain, and heart, and cause local destruction of the tissue. (It is possible that they break out in other organs and tissues and make similar lesions, but this case did not show them in either kidney or bladder after careful search.)

(4.) The capsule of the encysted trichinæ is formed of connective tissue which surrounds the whole of the invaded fiber.

(5.) This case shows a fatty change in the liver, but does not show a fatty change in the heart or kidneys or an enlargement of the spleen, which lesions have been reported by other observers.

[In conclusion I want to thank Dr. Mallory for his corrections and interest in this paper, Dr. Southard for his help on the brain lesion, and Dr. Orton for his photomicrographs.]

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PLATE XXX.

(*The arrow in each figure points to the trichina embryo.*)

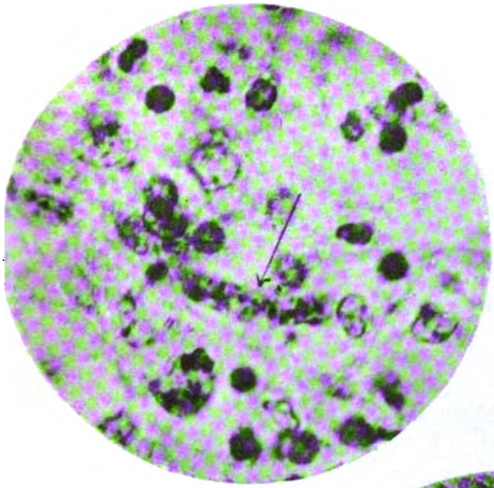
FIG. 1. Part of a trichina embryo free in a lymph sinus of a mesenteric lymph node. x 1,000.

FIG. 2. Part of a trichina embryo free in a hemorrhagic area in the lung. x 500.

FIG. 3. Local lesion in the heart. The trichina embryo can just be seen. x 500.

FIG. 4. Trichina embryo free in a liver sinusoid. x 1,000.

FIG. 5. Trichina embryo in the brain. x 1,000.



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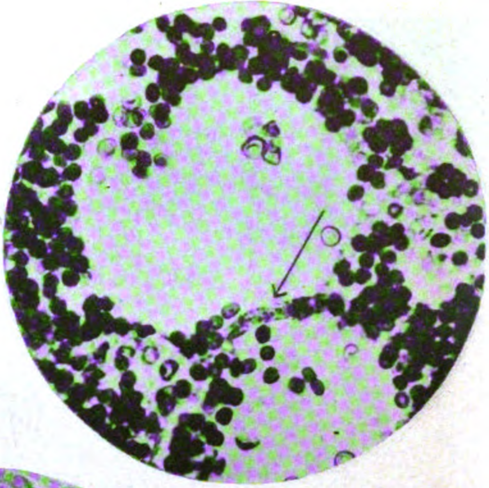

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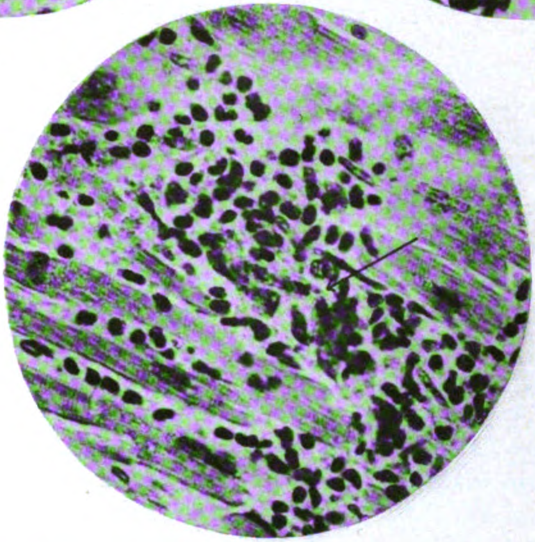

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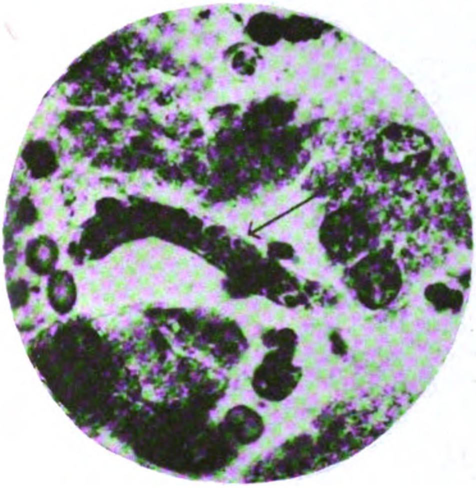

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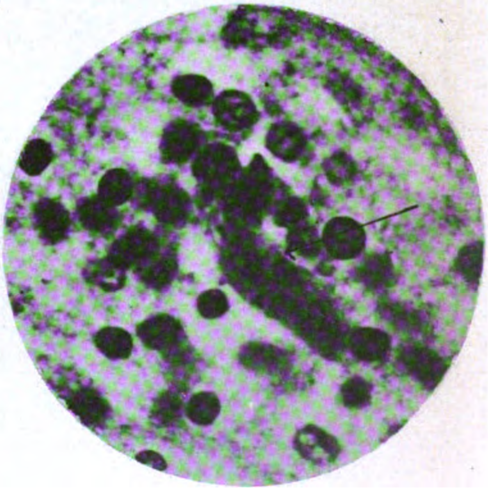

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C. Frothingham.

Trichina spiralis.

CONCERNING HEMOFLAGELLATES OF AN AFRICAN FISH (CLARIAS ANGOLENSIS).*

BY

THE LATE
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AND
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(*From the Runcorn Research Laboratories of the Liverpool School of Tropical Medicine.*)

I. TRYPANOSOMES.

Two "mudfish" (*Clarias angolensis*) caught at Leopoldville in the Congo Free State on December ninth and December thirtieth, 1903, had actively motile trypanosomes in their blood that may be divided into three types: a small, a medium, and a long form. The small type was identical in both, the medium was present in both, but with slight differences, and the long form was present only in the fish of December ninth. The small form was very frequently seen in the fish of December thirtieth, while in the fish of December ninth only four were present in a total of ninety-nine parasites. The medium form, in the fish of December thirtieth, was less frequently met with than the small form, but the type was often seen in the fish of December ninth. Of the long forms only thirteen were seen. All the forms were characterized by the possession of a large four-lobed blepharoplast, situated at, or very near, the posterior extremity, and by a clear space about the nucleus. The blepharoplast apparently consisted of four darkly-staining granules of equal size embedded in a matrix. In a few specimens there were, perhaps, more than four granules in the blepharoplast, but in these cases it was never possible to be certain of the exact number. The three forms of parasites seemed but different variations of one species, as graduations between all forms could be seen.

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The small type was readily distinguished by its size, its narrow body, its relatively long nucleus — situated more anteriorly than in the other two types and with its long diameter parallel to that of the body — and by its faint staining reaction. The blepharoplast was oval, or round, large and distinct (measuring 1.13 by 0.75 μ) and was situated at the posterior extremity. The undulating membrane was well developed, having a width of 0.75 μ. The long, oval nucleus was situated at the junction of the middle and anterior thirds of the body. It was granular in structure and contained a fibrous network on which were seen eight to sixteen chromatin granules and one darker granule — the karyosome. The body protoplasm was reticular and contained a few violet-staining granules. Occasionally parasites were seen in which the body was filled with these coarse chromatophilic granules. Superficial longitudinal striations to the number of five could be seen near the nucleus of two slightly injured parasites. Division forms were very numerous in this type (even when but four were seen in the fish of December ninth, one of them was dividing). They followed the usual method of longitudinal division. In the division of the blepharoplast, the posterior lobe on the concave side of the parasite moved behind the other posterior lobe; the axis of the posterior lobes thus became longitudinal while that of the anterior lobes remain transverse. The two posterior lobes then separated from the anterior lobes and their axis became transverse again.

The medium type was characterized by its size, and its darker staining reaction. The blepharoplast was large, oval or round, distinct and measured about 1.5 by 0.75 μ. It was situated at the posterior extremity, or from 1 to 1.5 μ anterior to it. The flagellum was relatively longer than in the short form. The undulating membrane in parasites from the fish of December thirtieth was well developed, and wound in numerous folds around the body; it was 1.5 μ wide. In trypanosomes from the fish of December ninth, the undulating membrane was poorly developed, had a few folds and was not more than 1 μ in width. The nucleus was round to oval, its long diameter was at right angles to that of the body, and it occupied the whole width of the parasites. It was granular in structure and contained a fibrous network on which were seen sixteen to twenty-four chromatin granules and one or two darker granules — the karyosomes. The body was more darkly stained in the fish of December thirtieth. Superficial longitudinal striations to the number of seven were counted near the nucleus. Divisional forms were few in this type and followed the usual method of longitudinal division.

The long type was characterized by its size, its tapering extremities, its comparatively short flagellum and by the possession of from one to four vacuoles just anterior to the blepharoplast. The blepharoplast was large, oval, or round, and situated 2 μ from the posterior extremity; its measurements were the same as those of the medium type. The nucleus had the same characteristics as that of the middle type, except that it did not quite occupy the width of the body. The protoplasm was the same as in the medium type, except for the above-mentioned vacuoles which were always present. Eight superficial longitudinal striations were present, and these were seen to cross the nucleus. No divisional forms of this type were seen.

The measurements of the three types were as follows:

	Small.	Medium.	Long.
1. Posterior extremity to center of blepharoplast	0 μ	0 to 15 μ	2 μ
2. Center of blepharoplast to posterior border of the nucleus	12.75 μ	14.25 μ	25 μ
3. Length of nucleus	3 μ	3.75 μ	45 μ
4. Anterior border of nucleus to anterior extremity of the body	6.75 μ	12 μ	19.5 μ
5. Length of the free flagellum...........	12 μ	13.5 μ	10.5 μ
6. Width of the body, without undulating membrane, at its widest part	1 μ	3 μ	4.5 μ
7. Usual, total length of the parasite......	34.5 μ	45 μ	61.5 μ

Although the trypanosome described by Montel[1] closely resembles our large form, we have seen no account in the literature of any parasite morphologically identical with those described above.

II. A SPIROCHÆTA.

In the blood of the fish of December thirtieth a spirochæta-like organism was found which could be identified with no previously described spirochæta.

It had a hair-like body; its length was usually 18 μ; its breadth, at the widest part, was 0.6 μ. It was widest at one extremity or towards the middle; in the latter case, the parasite tapered toward both ends, but one extremity was always much thicker than the other. The wider end was rounded, while the other end was drawn out to a fine and slender point. The parasite usually occurred singly and lay in a simple curve forming three-quarters of the circumference of a circle (Plate XXXII., Figs. 1 and 2). Other forms — coils (Fig. 3), compound curves (Figs. 4, 9–12) and spirals (Figs. 5–8) — were seen as illustrated. The coils were always formed by the slender end of the parasite. The spiral forms were long, measuring from 24–27 μ, and in some of these forms there was a suggestion of a membrane (Figs. 5 and 6). The parasites were also seen in pairs and in groups of from three to many individuals as shown in Figs. 13, 14–16. These organisms seemed to consist of a deeply-staining core and a lightly staining periplast. Many of them stained irregularly by modifications of Romanowsky's method. The lighter stained areas (Fig. 2) occurred at irregular intervals and were of irregular extent. The periplast could be seen at these lightly stained areas and it was continued to form the slender extremity from which the core was also absent. Occasionally granular forms were seen in which the protoplasm of the core apparently arranged itself into small granular masses, but no regularity could be detected in the arrangement of these granules. Undoubted multiplication forms were not seen.

For descriptive purposes we propose for this organism the name of *Spirochæta jonesii*.

LITERATURE.

Good lists of the publications on the trypanosomata of fishes will be found in the works of Laveran and Mesnil (Trypanosomes et Trypanosomiasis) and of Mense (Handbuch der Tropenkrankheiten).

The following are important papers not mentioned in these volumes:

1. Montel, 1905. Trypanosoma d'un poisson de Cochinchine. Comptes Rendues de la Societe de Biologie, i, 1016.

2. Lebailly, 1906. Recherches sur les Hématozoaires des Téléostéens marins. Thése Fac. Med. Paris, 1906, p. 76. Archiv. de Parasitologie, x.

3. Lebailly, M. C., 1905. *Trypanosoma bothi* (Barbue) n. sp. Comptes Rendues de la Societe de Biologie, ix, 304.

4. Brumpt, 1906. Sur quelques especes nouvelles de Trypanosomes parasites des Poissons d'eau douce. Comptes Rendues de la Societe de Biologie, lx, 160.

5. Brumpt, 1906. Mode de transmission et evolution des Trypanosomes parasites des poissons. Comptes Rendues de la Societe de Biologie, lx, No. 4, 162.

DESCRIPTION OF PLATE XXXI.

FIG. 1. Small form, showing arrangement of the chromatin granules of the nucleus and of the granules of the blepharoplast.

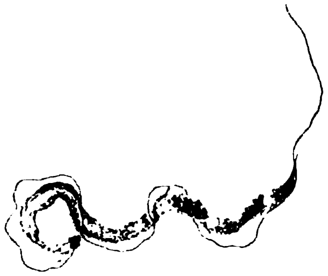
FIG. 2. Small form, showing early stage of division. The chromatin granules of the nucleus have gathered into two masses, and the granules of the blepharoplast have changed their position.

FIG. 3. Medium form, showing arrangement of the chromatin granules of the nucleus and of the granules of the blepharoplast.

FIG. 4. Medium form, showing striations.

FIG. 5. Long form, showing arrangement of the chromatin granules and karyosome of the nucleus, the longitudinal striations, the granules of the blepharoplast and the vacuoles anterior to it.

The drawings of these are based on careful measurements. They represent the appearances noted with a magnification of twenty-two hundred and fifty diameters (Zeiss, two millimeters apochromatic objective, No. 18 eyepiece).



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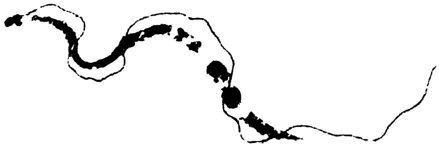

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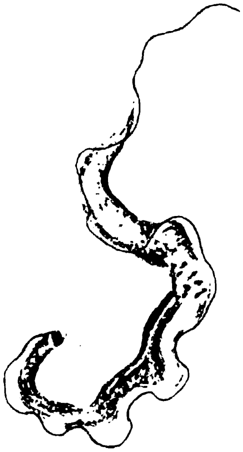

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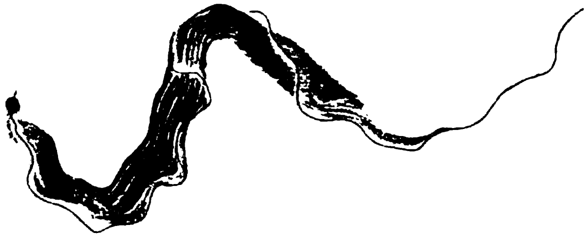

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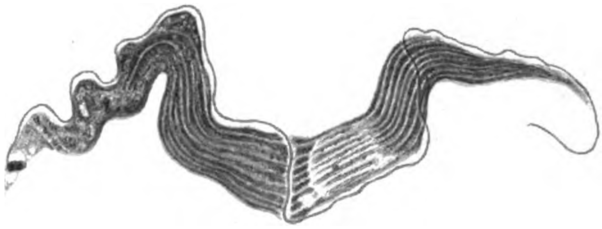

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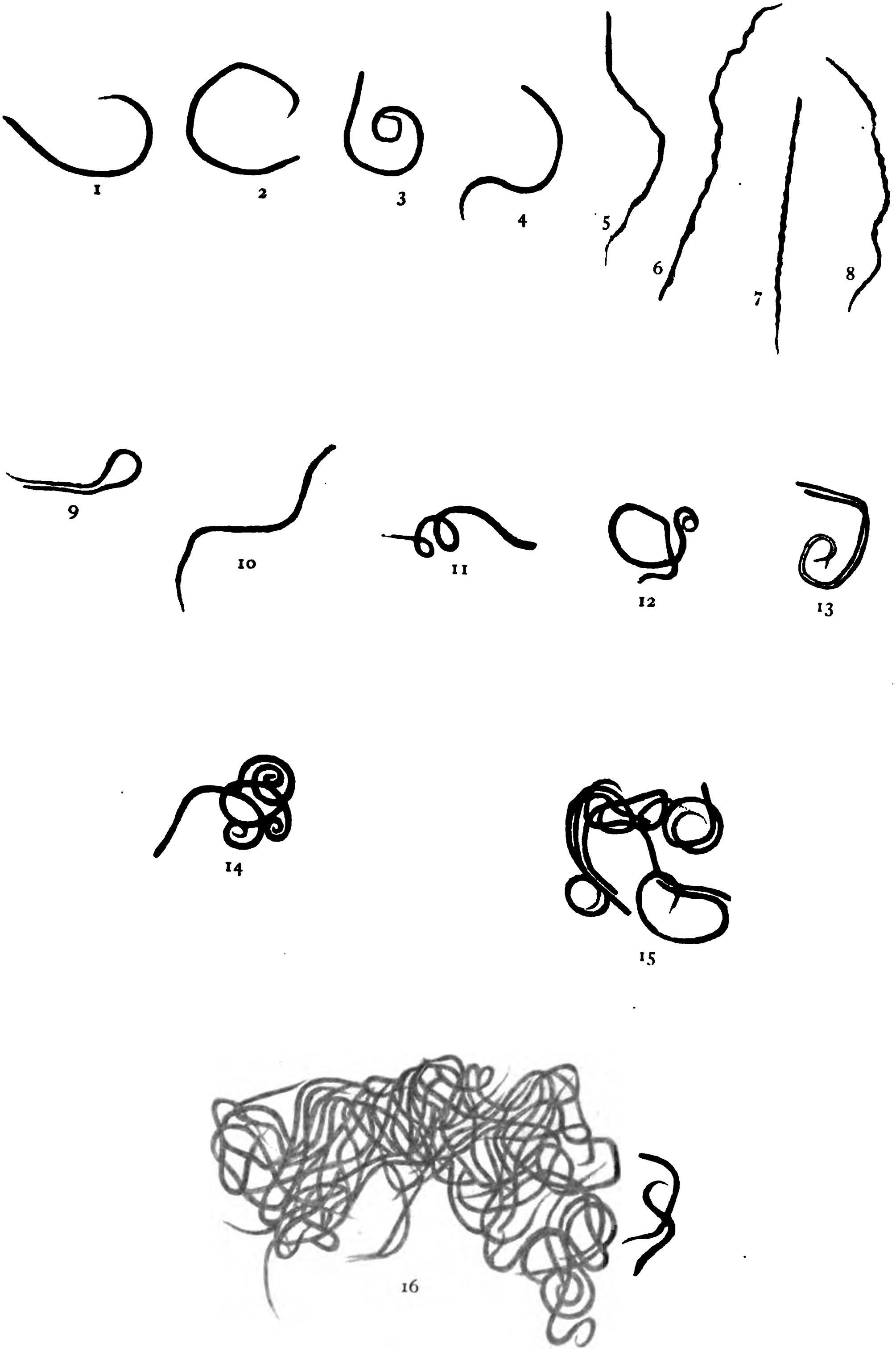
Dutton. Todd. Tobey.

Hemoflagellate.



Dutton. Todd. Tobey

Hemoflagellate

NOTES ON B. COLI IN DRINKING WATER. ATYPICAL FORMS FOUND IN THE SAME, AND SOME OBSERVATIONS CONCERNING A PECULIAR "RED REACTION" WHEN NAOH IS ADDED TO THE CULTURES.*

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Since the chemical analysis does not always furnish sufficient evidence of the pollution of water, and because the pathogenic bacteria are found by preference in water chemically pure, the hope of bacteriology to find methods capable of revealing the presence of specific disease germs has not been without foundation. While this problem may be regarded as solved in the case of the spirillum of Asiatic cholera, it is far from solution in the case of the typhoid bacillus. All efforts made in this direction have been in most of the cases negative. Realizing the difficulties inherent in this problem, on account of the presence of the colon group in polluted water, attention has been given to the latter, as an indicator of pollution, the practice being to condemn all waters containing the colon group of organisms; on the other hand, the wide distribution of this group, its rapid multiplication outside of the animal body and especially its occurrence in the excreta of mammals and birds as well as in the intestines of man, has led one or several bacteriologists to regard B. coli of little value as an index of sewage contamination. It does not seem justifiable to assume that the excreta of animals are harmless to man and that a water so polluted could be desirable for drinking purposes. Further, it should not be regarded as a fair comparison when two waters, both containing the colon bacillus, in one of which it was necessary to experiment with one liter to detect the organism, and in another with only one cubic centimeter or a fraction of it; it is logical to assume that it is not the mere presence of B. coli that should tend to condemn a water, but also its relative abundance, and the conditions under which it is found.

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Before entering upon the technic for the study of the colon group it is desirable to emphasize the fact that though the isolation of B. coli from water is merely a matter of laboratory practice and presents but little difficulty, the morphological and biological characters of the colon group are so varied that not seldom do we meet with varieties in which a positive diagnosis requires the utmost care, and in some cases it is almost impossible. In view of these changes some authors have been inclined to admit different varieties, all belonging to the same group.

Booker, in his exhaustive study relating to bacteria found in feces of infants suffering from summer diarrhea, has isolated seven varieties which resemble B. coli communis in morphology and growth on agar, neutral gelatin, potato, milk, and in their gas production or reaction with litmus. Gilbert came to the conclusion that there are five types of this bacillus very closely related to the colon group: (1) A bacillus which differs from the colon bacillus in being non-motile; (*a*) B. lactique of Pasteur; (*b*) B. of endocarditis of Gilbert and Lion. (2) Bacillus which does not give the reaction of indol. (3) Bacillus which does not ferment lactose. (4) Bacillus which is not motile and does not ferment lactose. (5) Bacillus which is not motile and does not give indol reaction nor ferment lactose.

Fremlin found the fermentation of glucose and coagulation of milk the most common characteristic of B. coli, the most active movements are said to be observed in the bacillus from man, while that isolated from rabbits shows scarcely any movement.

Finally, Smith goes so far as to regard the fermentation of sugar sufficient evidence of the presence of colon bacillus in water, regarding any further study unessential, and though B. enteritidis, B. typhi murium and B. choleræ suis present the same reaction, they are as seldom found in water as B. typhosus itself. Further on he gives a certain proportion of gas production, and the relation between the H and CO_2 in the fermentation as peculiar to B. coli.

All these statements may be regarded as correct for each separate species of the colon group. The same variation has been met also in our investigations, and no doubt the marked variations which the colon group presents has led some to regard B. coli and B. typhosus as but varieties of one and the same species, which problem it is not our purpose to discuss. Our present work is limited to the consideration of the morphological and biological characteristics of B. coli as described by the majority of investigators, and at the same time to consider the changes which the colon group presents, especially in cultures recently isolated from water.

Abbott, in his "Principles of Bacteriology," gives the following points as characteristic of the colon bacillus:

> Facultative anaërobic bacillus, with no peculiar morphological features that can aid in its identification; motile in the majority of cases; by Löffler's method of staining flagella can be demonstrated; fecal odor in old bouillon cultures, does not liquefy gelatin, coagulates milk, and when this contains litmus tincture, the blue color is changed to red in twenty-four hours. It ferments glucose; in lactose litmus agar the colonies are pink; produces indol, and reduces nitrates into nitrites, and does not stain by Gram's method. Likewise the same characteristics are described by Beson, Sternberg, etc. McFarland, in his Text-book of Pathogenic Bacteria, gives a most valuable table on differential diagnosis of the most common organism found in water. He describes B. coli as above, adding to it the amount of gas produced and the relation of $\frac{H}{Co_2} : \frac{2}{1}$ as described by Smith.

Reaction of the medium. — Without going into the details of the preparation of the culture medium and solutions usually employed for the detection of the colon group, a description of which can be obtained from any text-book on bacteriology, we desire only to relate some observations regarding the reaction of the culture medium. Some investigators recommend the acidity of 1.0–1.0 per cent of hydrochloric acid, sulphuric acid, or of the normal acid of meat (sarcolactic acid) with the claim that such reaction is favorable to the growth of the colon group. According to our observations it seems that such acidity is not essential, and, in some cases, undesirable, as there are other bacteria closely related to the colon group, but not identical, to which such acidity is not only unfavorable but detrimental.

In the study of a number of stab cultures on gelatin of 1.0 per cent to 1.5 per cent acidity, which did not show any perceptible liquefaction after the tenth to fourteenth day, and were regarded as belonging to the colon group, a new inoculation into neutral gelatin, the liquefaction of the medium was observed from the fifth to tenth day. Further, on the neutral gelatin the cholera spirillum showed a slight liquefaction as early as the second day, and this was complete after the tenth day, while on acid gelatin the cholera spirilla began to liquefy the medium very slowly (after the fifth day), and even after two weeks the liquefaction was only slight though distinct. B. coli remained unliquefied. It was also noted that in most of the cultures (as well as of B. coli) the growth was more luxuriant on the neutral gelatin.

This experiment we believe proves conclusively that though the colon type grows well on acid media, this acidity may inhibit some of the biological reactions (the liquefaction of gelatin) of debilitated cultures isolated from water, closely related to B. coli but not identical with it, and that a neutral medium is to be prepared.

Method. — In the isolation and identification of B. coli there are two methods commonly used; one is the presumptive test in which a series of samples of the water, in suitable dilution (.001, .01, 1., etc., 10 cubic centimeters), is added directly to the dextrose broth in a Smith fermentation tube and incubated for twenty-four hours at 37° C. On measuring the gas the formation of from twenty-five to seventy per cent of the capacity of the closed arm, and, if of the gas approximately one-third is CO_2 and two-thirds hydrogen ($\frac{H}{CO_2}$: $\frac{2}{1}$), the reaction is considered as typical. If the amount of gas is between ten and twenty-five per cent or more than seventy per cent, or if the percentage of CO_2 is greater than forty per cent the reaction is considered atypical, while if no gas forms, or less than ten per cent, the test is considered negative.

This test is very rapid, enabling one to make a diagnosis in the short time of twenty-four hours, but as the fermentation of dextrose is not peculiar to the B. coli alone, and further, as the amount of gas produced and the proportion of H to CO_2 evolved is not constant for B. coli in all cases, the importance of such a test has its limitations, and in some cases may give erroneous results, as I hope to be able to show in a further paper. Cases have come under my observation in which samples of water giving high bacterial counts, and containing B. coli, the incubation of one cubic centimeter in dextrose broth fermentation tubes not only gave an amount of gas below ten per cent, but failed completely to produce any fermentation. The other methods employed for the detection of the colon bacillus are the same as for the typhoid bacillus. Rodet was the first to point out that this bacillus is capable of growing at the temperature of 45° C. Chantemesse and Widal found both bacteria capable of growing in a culture medium containing carbolic acid. Vincent, making a combination of these two principles, placed the culture at 41°–42° C. in a medium to which carbolic acid was added. Péré, upon the same principle, gives a method which permits the experiment to be made with a large quantity of water. Foote recommends agar plates at 37° C. Wurtz, agar to which lactose has been added, and tinged with litmus. The colonies upon such plates divide themselves into two groups; those which attack the lactose and produce acid, imparting a pink color to the colonies, and those which do not act upon the lactose. The colon group belongs to the former, and the typhoid bacillus to the latter. Finally, the addition to the culture of a certain amount (two per cent) of Parietti's solution, which permits the growth of B. coli to the exclusion of many other bacteria.

As will be readily understood, each one of these principles has its own merit; not one of them alone will suffice; the addition of carbolic acid or Parietti's solution though detrimental to a great number of water bacteria, will also permit the growth of many others besides B. coli. Likewise the pink colonies on litmus lactose agar and the fermentation of sugar is common to other than the colon group.

Therefore, in view of such peculiarities, a combination of these principles is not only advisable but absolutely necessary.

The routine technic consists in plating a given quantity of the water, one cubic centimeter or a fraction of it, according to the degree of pollution in Wurtz litmus lactose-agar, and after an incubation of eighteen to twenty-four hours at 37°–40° C. The characteristic pink colonies resembling B. coli are carefully counted and a number of them inoculated in dextrose broth fermentation tubes, and placed in the incubator at 37°–40° C. After twenty-four to forty-eight hours the tubes not fermented are discarded, while the fermented ones are further inoculated in Smith sugar-free bouillon, or Dunham's solution for the production of indol, and nitrate-peptone solution, for the reduction of nitrates into nitrites; inoculations of the milk and gelatin stab cultures are also made and placed in the incubator, the gelatin at 19°–20° C., and the others at 37°–40° C. The amount of gas in the fermentation tubes after twenty-four and forty-eight hours is observed and the relation of H to CO_2 also noted.

After the fifth day all the cultures except the gelatin are examined, the coagulation or non-coagulation of the milk, and the production or non-production of indol is determined by the Salkowski reaction. The reduction of nitrates into nitrites by the Naphthylamine sulphanilic acid reaction; after the tenth day the character of the gelatin stab cultures and the presence or absence of liquefaction of the gelatin also is noted.

At the same time that the biological reactions are studied, the character of the culture is determined — if bacillus, its size, motility, number of cilia, whether or not it forms spores, etc., and finally, whether decolorized by Gram's method.

The determination of the number of B. coli per cubic centimeter is merely a matter of calculation based upon the positive results obtained. The total number of pink colonies resembling B. coli in the original plate will represent a given quantity of the water examined.

As a matter of fact, in the course of the examination, a typical reaction is not always obtainable. This may be attributed to a variation which bacteria recently isolated may present, due, very probably, to changes produced during the long standing in the water under unfavorable conditions; or, these changes can be attributed to the great variation which the colon group itself presents as stated above; therefore, in view of these peculiarities it is desirable to consider these variations in order to arrive at a more precise diagnosis. This article is presented merely as a contribution to the study of the colon group. The writer attempts to consider only the variations which occur and the difficulties in the isolation and recognition of B. coli in drinking water. These observations include a great number of experiments in which comparisons were made of B. coli cultures obtained in this country and abroad.

1. The amount of gas evolved during the fermentation (25–70 per cent) and the relation of two parts of hydrogen to one of carbon dioxide($\frac{H}{CO_2}:\frac{2}{1}$) as given by Smith and recommended by some bacteriologists was found only in a few cases; in some instance it was $\frac{H}{CO_2}:\frac{4}{1}$; while in others just the reverse, $\frac{H}{CO_2}:\frac{1}{4}$. Such cultures, it is understood, did not only ferment dextrose, but also presented all the characteristics of the colon group; that is, produced indol, reduced nitrates, coagulated milk, did not liquefy gelatin, etc., and in an average made from over five hundred cultures isolated from the Delaware and Schuylkill rivers the proportion was $\frac{H}{CO_2}:\frac{3}{2}$. Likewise, in some cases, the total amount of gas evolved during twenty-four and forty-eight hours at 37°–40° C. was as low as ten per cent, and as high as ninety per cent; it should also be noted that in one instance twelve cultures isolated from the Schuylkill river gave the proportion of $\frac{H}{CO_2}:\frac{3}{1}$.

Further on in experiments made with a number of cultures obtained from the Laboratory of Hygiene of the University of Pennsylvania, though the proportion of $\frac{H}{CO_2}:\frac{2}{1}$ was obtained in some cases, a variation was noted not only among the several cultures, but also in the same culture under the same conditions, and in one instance it was $\frac{H}{CO_2} : \frac{1}{1}$. Similar variations were also noted with a culture obtained from the laboratory of the Board of Health of Philadelphia. Out of eighteen experiments made with different cultures obtained from the Institut Pasteur of Lille, France, in four the proportion was $\frac{H}{CO_2} : \frac{2}{1}$, in one instance it was $\frac{H}{CO_2} : \frac{4}{1}$, and in the remainder the excess of hydrogen was always constant, and though a variation was also noted, the average showed the proportion of $\frac{H}{CO_2} : \frac{2}{1}$. Therefore, from the above results it may be concluded that the excess of hydrogen in the proportion of two to one, is more apt to be met with typical laboratory cultures of B. coli; but as such a proportion was not a constant occurrence, it is not so important for diagnosis, provided that the other reactions occur together with the characteristics of the colon group. This should be the rule, especially in cultures recently isolated from water, which, as stated above, are more apt to present variations. Likewise, any culture giving a percentage of gas below ten or above ninety should not be discarded, that is, considered as not belonging to the colon group, but it must be suspected as such and further examined for the other characteristics of B. coli.

2. The variation in intensity which the reaction of indol presents is well known; in some cases it is typical, in others only a slight diffuse pinkish coloration is observed at the bottom of the tube, while in others it requires long standing, or gentle heating to bring about the reaction. The long standing and gentle heating of the culture, though advised by the majority of writers, in my hands has given unsatisfactory results; it may be emphasized that by heating I have been able to produce a typical reaction in the typhoid culture, which was colorless before the operation, and though the production of indol by the typhoid bacillus has been claimed by some bacteriologists it must be remembered that at present it is regarded as one of the points in differentiation from the B. coli. I have further observed by long standing

a slight reddish coloration, not only in typhoid cultures, but also in the uninoculated control tubes containing the sterile meat sugar free bouillon.

By using the procedure recommended by McFarland and Small, which consists of the concentration of the reaction in the form of a ring, by adding the reagents with the least possible disturbances of the medium, with all the cultures studied with the sugar free meat bouillon and examined after the fifth day of incubation at 37° – 40° C., I have been able to obtain one, two, or even three rings; the reaction was typical and almost immediate after the gentle addition of the sulphuric acid and potassium nitrate solution. In case of doubt it is not so important to lay stress upon the intensity of the reaction, and the condition under which an atypical reaction has been obtained, as it is to repeat the process with new media if necessary and as often as required. By the procedure of rejuvenation, I have succeeded, after some preliminary cultivation, in exciting the reaction from cultures which at first gave only a faint, and by no means typical reaction of indol. As a matter of precaution it is advisable to submit to the same test and under the same condition besides an uninoculated tube, also a culture of the colon and typhoid bacillus as control.

3. Regarding the coagulation of milk it will be observed that there is a constant relation between the production of pink colonies on litmus lactose agar and the coagulation of milk. In an observation made upon twelve hundred and fifty-two cultures, five did not coagulate the milk, four of them did not present the characteristics of B. coli and were not further examined, and the remaining cultures, though presenting all the other characteristics of B. coli, failed to coagulate milk; by preliminary cultivation, however, the milk was coagulated after two weeks incubation at 37° – 40° C., or, after one week by heating. Therefore, it seems unnecessary to test the coagulation of milk from a culture which gives a typical pink colony on litmus lactose agar plate.

4. In routine work it is not advisable to conduct the experiment with more than one cubic centimeter (50 cc.,

100 cc., etc.) to determine the degree of purity of a water, as more often gives contradictory results. In one experiment made with two waters, one containing twenty and the other fifty B. coli per cubic centimeter, a second experiment with fifty cubic centimeters of the same water, under the same conditions, failed to show any of the colon group. This was due very probably to a symbiotic process, detrimental to B. coli. In a number of experiments conducted along the same line, results similar to the above were met more frequently than was expected, and, though this is not always the case, it may be taken as sufficient evidence of the little value which the use of more than one cubic centimeter presents. To experiment with fifty, one hundred, or one thousand cubic centimeters no doubt has its scientific value, especially in the determination of the presence of the typhoid bacillus in water, which is so rare, but in the case of B. coli it is of little importance, if any; an accidental contamination is apt to take place during the manifold and prolonged manipulation which leads to condemnation of a water which properly should be regarded as safe.

5. The study of the morphological character of a culture, which in general routine work is so often entirely neglected, cannot be too highly recommended; a microscopic examination being absolutely indispensable in making a diagnosis. This is especially true with cultures in which the biological features are not entirely typical; such irregularity will be found to be due, in some cases, to bacteria closely related to the colon group, but not identical with it; and, in others, to impurity of the culture. Gram's method will aid considerably, and, in some cases, will determine the diagnosis.

The morphological examination of a number of cultures diagnosticated as B. coli, based upon the biological features alone, showed only eighty-four per cent to be such; ten per cent were mixed cultures and six per cent failed completely to show the characteristics of the colon group. Therefore, this experiment, I believe, will prove the necessity of making a microscopical examination of the culture under observation in order to make a diagnosis with certainty.

Before concluding, it is desirable to make some remarks regarding a peculiar "red reaction" which very often has given rise to error. If after the addition of a two per cent solution of sodium hydroxide to forty-eight-hour-old culture in the fermentation tubes, they are left aside, after two to four hours a slight reddish coloration is observed beginning at the surface, which gradually diffuses into the medium; later, the reaction becomes more intense and on the next day a pinkish or reddish-brown coloration of the liquid is noted in the bulb of some tubes, while others remain apparently unchanged. No doubt for sufficient reason, but perhaps with some misconception, this coloration is regarded as an indication that the culture under observation does not belong to the colon group. The literature seems to be very meager, and as I have not been able to secure any communication upon this peculiar reaction, I may be permitted to give some observations upon the subject.

If to a number of forty-eight-hour cultures isolated from water and grown in dextrose broth fermentation tubes, a two per cent solution of sodium hydroxide is added, it may be observed after twenty-four hours that some tubes present a coloration from a lemon to orange hue, reddish, or even distinct pink, while others remain unchanged. The reaction is limited to the bulb, usually. In other cases the reaction disappeared after the second, third, or fourth day, and in a very few cases it persisted longer.

A number of experiments conducted with cultures isolated from the water presenting this red reaction showed the coloration to be constant, while with other cultures, also isolated from the water, not giving the reaction, examined with the same medium and submitted to the same test, under the same conditions, in no case was the reaction obtained. Showing that the red reaction was peculiar to a certain kind of bacteria, the next step was to determine its relation to the colon group.

Out of forty experiments, conducted with six cultures of B. coli from different sources, in one instance only was a very slight yellowish-brown coloration (by no means characteristic) observed, while in the other thirty-nine the liquid remained always unchanged. Further, out of fifty experiments made with twelve cultures, not belonging to the colon group, but which gave the reaction, the coloration was constant in each case. This result shows that such reaction has no relation to B. coli, and that any culture presenting such coloration with NAOH solution can properly be discarded as belonging to the colon group; but it must be remembered that in none of the fifty experiments was a typical reaction of B. coli obtained. In this case, therefore, the coloration is of little importance. This, however, is not always the case, and not seldom do we find cultures which present the characteristic of B. coli in addition to the red reaction; in consideration of these it was desirable to investigate further upon the subject.

From a number of cultures isolated from water which presented the characteristic features of the colon group, and which by this peculiar red reaction with the solution of NAOH were rejected as such, some were selected and closely examined. Four successive experiments with such cultures gave the characteristics of B. coli; the red reaction was obtained each time also. Learning from the other experiments that such coloration was not related to the colon group, the result was contradictory and an impurity of the culture was suspected. Plates were made with the usual precautions, two colonies were isolated; further research with them showed the red reaction to persist, but failed to produce indol. This result proves that the culture under consideration was impure, containing no less than two organisms; one, the bacterium, which produces this peculiar red reaction on dextrose broth on the addition of a two per cent solution of NAOH, and the other, B. coli; therefore, according to this result, the negative diagnosis of B. coli in these cultures may be regarded as erroneous, and the red coloration in cultures which present also the characteristics of B. coli must be considered secondary in making the diagnosis.

In further studies upon this red reaction with NAOH solution the color acted as an indicator, disappearing on the addition of acid and reappearing with an alkali.

A number of cultures isolated from water showed the following biological features:

Litmus lactose agar plates, colonies pink in most cases, in a few instances the medium remained unchanged, dextrose was fermented, in most of the cases producing over ninety or even one hundred per cent of gas in forty-eight hours at 37° C.; in other cultures the fermentation was very weak, below ten per cent, or failed completely to attack the dextrose. Indol was produced in exceptional cases only. Nitrates were reduced to nitrites. Milk coagulated in most of the cases. Gelatin liquefied in some instances. There were also cultures very closely related to the colon group.

This variation may indicate that there is more than one species which possesses this property, and, as the red coloration was constant with certain bacteria, and absent in others, as showed in the above experiments, one is apt to suspect that this reaction may be employed as a differential diagnosis. It is not my purpose to go deeply into this subject which will be discussed in a future paper, but merely to point out the fact that the occurrence of the red reaction, upon the addition of NAOH solution, in cultures which at the same time give the characteristics of the colon group should suggest not to reject them as B. coli, but to further investigate the purity and character of the culture.

[In conclusion I desire to express my most sincere thanks to Monsieur Prof. A. Calmette, Dr. N. Gildersleeve, and Dr. Stewart for their kindness in furnishing me with cultures for this work.]

A.

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